Antisense Oligodeoxynucleotides: from the Bench to the Patient

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1. Introduction

Gene expression may be disrupted by a variety of methods. It is convenient to group the available technologies according to whether they are targeted to the gene itself, e.g. homologous recombination, or to the gene's transcriptional product, messenger RNA. Among the perturbation strategies which are RNA directed, the most widely employed are catalytic RNA molecules or ribozymes, and the so-called "antisense" oligodeoxynucleotides (1). The latter are short nucleotide sequences of DNA which are synthesized as exact reverse complements of the desired mRNA target's nucleotide sequence. In theory, the antisense DNA molecule can only hybridize in a stable manner with its mRNA target. Once the RNA-DNA duplex has been formed the, translation of the message is prevented, and/or destruction of the molecule by RNAse H is promoted. Since the earliest attempts by Zamecnick and Stephenson to inhibit Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide (2), antisense DNA compounds appeared an attractive tool not only for investigations of normal and pathological gene functions, but also as potential therapeutic agents in a spectrum of pathologic processes ranging from viral infections to neoplastic disorders.

The pioneering observations on point mutations of the ras transforming genes isolated from epithelial neoplasia (3), and the subsequent elucidation of two other common modalities of oncogene activation in cancer cells, amplification (e.g., erb-B2 amplification in breast and ovarian cancers) and translocation [e.g., juxtaposition of the bcr and c-abl genes in chronic

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myelogenous leukemia(CML)], have led to the realization that the tumor-specific abnormalities generated by the activation of protooncogenes can be exploited for nucleic acid-targeted therapy. The potential for such highly specific targeting contrasts with the mechanism(s) of action of conventional anti-cancer chemotherapeutic agents which block enzymatic pathways or randomly interact with nucleic acids irrespective of the cell phenotype. Anti-cancer chemotherapeutic agents exploit differences in biochemical or metabolic processes (e.g., growth rate) between normal and cancer cells for the preferential killing of neoplastic cells over normal cells. In contrast, antisense oligodeoxynucleotides exploit the presence of genetically defined characteristics that distinguish neoplastic cells and are responsible for their growth advantage over normal cells. In recent years, the antisense strategy for cancer therapy has progressed from *in vitro* culture studies, to investigations on animal models, and now to clinical studies. We describe here the current state of progress toward gene-directed antisense-based cancer therapy targeting BCR/ABL oncogene, primarily from the viewpoint of initial proof-of-concepts studies on aminal models of human leukemias and phase I clinical investigations.

2. Targeting bcr-abl mRNA in a SCID mouse model of Philadelphia[1] leukemia

The ideal strategy for the treatment of leukemia would seek to selectively eliminate leukemic cells and to restore normal hematopoiesis. An example of such rational drug design is the targeting of bcr-abl transcripts found in leukemic patients carrying the Philadelphia chromosome translocation (4-6), which has attracted considerable attention as a paradigm uniquely suited to the antisense concept. The pathogenetic role of the bcr-abl genes in CML has been strongly suggested by the appearance of CML-like syndromes in mice bearing bcr-abl constructs (7-9). Synthetic oligodeoxynucleotides complementary to the junction of bcr-abl transcripts produced from the splicing of either the second or the third exon of the bcr gene to the second exon of c-abl were shown to suppress Philadelphia[1] leukemic cell proliferation *in vitro* and to spare the growth of normal marrow progenitors (10). A prerequisite for the *in vivo* utilization of antisense oligonucleotides as anti-cancer drugs is the development of animal models of human malignancies that mimic the natural course of the disease in patients. Unlike other types of human neoplasia, leukemic cells obtained directly from marrows of patients can be transplanted into immunodeficient SCID mice and show a pattern of leukemic spread reminiscent of that observed during the natural course of the disease (11). Initial *in vivo* findings in SCID mice injected with Philadelphia[1] BV173 and systemically treated with a nuclease resistant 26-mer b2/a2 antisense phosphorothioate oligodeoxynucleotides ([s]ODNs) at 1 mg/day for 9 consecutive days have been very encouraging (12). The treat-

ment led to marked decrease in three different measures of leukemia burden: percentage of CALLA-positive cells, number of clonogenic leukemic cells, and amounts of bcr-abl transcripts in mouse tissues. Similar studies conducted on SCID mice carrying Philadelphia[1] cells directly taken from a patient with CML in blast crisis confirmed the ability of bcr-abl antisense [S]ODNs to temporarily suppress the spreading of leukemia (13).

3. Targeting of two cooperating oncogenes/protooncogenes

Although bcr-abl is clearly the most rational therapeutic target for patients with CML, theoretical considerations suggest that it might be wise to investigate the efficacy of other molecular targets in this disease as well. The most significant of these concerns include the possibility that some CML stem cells might persist after exposure to bcr-abl targeted antisense because they do not express the bcr-abl mRNA (14). For this reason, and because the utility of bcr-abl antisense is restricted to CML patients, we have been examining the utility of other molecular targets. On the other hand, the partial anti-tumor effect of antisense oligodeoxynucleotides *in vivo* may reflect their inadequate uptake by the leukemic cells or an inefficient treatment schedule. Accordingly, repeated oligodeoxynucleotide injections may prolong survival or even cure leukemic mice if the tumor burden is greatly diminished. Alternatively, a "cocktail" of oligodeoxynucleotides or a combination of these agents with low doses of conventional antitumor chemotherapeutic agents might enhance the therapeutic effect. In this regard, we have been particularly interested in the c-myc gene.

Antisense oligodeoxynucleotides targeted against the proto-oncogene c-myc may augment the therapeutic effects of bcr/abl antisense oligodeoxynucleotides. The proto-oncogene c-myc was first associated with human malignancies because of its involvement in the chromosomal translocation of Burkitt lymphomas (15,16), and its amplification in tumor cell lines (17-19). Subsequently, the demonstration that c-myc expression is induced by mitogens and by platelet-derived growth factor (20), and that its constitutive expression partially abrogates growth factor requirements in growth factor-dependent cells (21,22) formally established the relevance of such gene in the regulation of cell proliferation.

Antisense oligodeoxynucleotides targeted to the initiation codon and downstream sequences of the human c-myc mRNA inhibited proliferation of normal T-lymphocytes and myelogenous leukemia line HL-60 (23-25). In the present context, several lines of evidence such as the synergism of c-myc and v-abl in transgenic models of plasmacytomas (26), and the selective enhancement of c-myc expression in myeloid hematopoietic cell lines constitutively expressing v-abl (27), suggest cooperation of bcr-abl and c-myc in transformation of hematopoietic cells. More recently, it was shown that a dominant negative Myc protein blocks the transformation induced by v-abl and BCR-ABL (28), and that c-Myc is required for the proliferation of CML cells (29), raising the

possibility that c-myc is a downstream effector of bcr-abl. In addition, trisomy of chromosome 8 (on which c-myc is localized) has been detected in a cohort CML of patients in blast crisis (30). Together, these findings suggest that the combined use of bcr-abl and c-myc antisense oligodeoxynucleotides might lead to enhanced therapeutic effects in SCID mice injected with Philadelphia[1] leukemic cells. In this regard, preliminary evidence suggests that, in combination, bcr-abl and c-myc antisense [S]ODNs exert a synergistic antiproliferative effect, *in vitro* and *in vivo*, using BV173 (31) and CML-BC primary cells (13), at concentrations at which individual [S]ODNs were only partially effective or completely ineffective. The reasons for the synergistic effect are not fully understood; it seems likely that, especially *in vivo*, antisense ODNs reach a plateau in their ability to downregulate gene expression which is not sufficient to completely block cell proliferation. Targeting of a second oncogene involved in the disease process may arrest the growth of cells that escaped the inhibitory effect associated with individual gene targeting. Alternatively, the downregulation of gene expression by single antisense [S]ODNs at relatively low concentrations reached *in vivo* might be insufficient to inhibit cell proliferation, whereas "partial" inhibition of two cooperating oncogenes might induce a more permanent block in the ability to proliferate. Although the importance of c-myc for normal cell proliferation raises the issue of undesirable side effects associated with c-myc antisense ODNs administration, the use of such compounds at non-toxic concentrations together with antisense ODNs such as bcr/abl, which target leukemia-specific sequences, did not cause evident toxicity in mice. A similar strategy might achieve, also in humans, enhanced therapeutic efficacy with minimal adverse effects.

4. Oncogene-targeted antisense oligodeoxynucleotides: potential clinical applications in hematological malignancies

In principle antisense oligodeoxynucleotides can be added to the armamentarium of anti-leukemic drugs and utilized for *ex vivo* or systemic treatment of leukemia.

Among the various experimental applications of autologous cell therapy, bone marrow purging has had a definite place in the treatment of several neoplasms, including acute and chronic leukemias (32). The marrow is cleansed of leukemic cells by a variety of agents that include immunological reagents (33) and chemotherapeutic drugs (34,35), and then is reinfused in patients treated with ablative chemotherapy. Theoretically, antisense oligodeoxynucleotides targeted against an oncogene that confers a growth advantage to leukemic cells should prove therapeutically useful and, most importantly, more selective than conventional chemotherapeutic agents in killing leukemic cells while sparing normal progenitor cells. However, several issues must be addressed before devising effective protocols for *ex vivo* use of antisense oligodeoxynucleotides in therapy. One issue relates to the half-life

of the mRNA target and, in consequence, to the time of incubation of marrow cells in the presence of oligodeoxynucleotides. For example, the half-life of Myc protein (10-30 min) is considerably shorter than that of $p210^{bcr/abl}$ protein (18-24 h), suggesting that a 24-48 h incubation of marrow cells might be adequate if the target is c-myb mRNA, but not bcr-abl mRNA. Another issue relates to the potential benefit of enriching hematopoietic progenitor cells before the *ex vivo* treatment to compensate for the relatively low proportion of clonogenic cells in marginally manipulated marrows. The selection of such enriched progenitor cell populations (e.g., $CD34^+$ cells) could also offset the likely differential uptake of oligodeoxynucleotides among marrow cells, which might result in ineffective targeting of leukemic cells. Last but not least, it must be remembered that the outcome of any purging approach for leukemia treatment is inextricably linked to the ability of the *in vivo* preparatory regimen to cleanse the patient of leukemic cells. It is straightforward that the cleanest autograft will rapidly become contaminated by viable residual leukemic cells left alive in the host marrow.

A protocol for *ex vivo* purging of $CD34^+$ enriched CML marrow cells with bcr-abl antisense DNA followed by autologous transplantation is now ongoing at the University "La Sapienza", Institute of Hematology, Rome, Italy.

The first phase I safety study of any antisense agent given systemically by i.v. infusion was begun at the end of 1992 at the University of Nebraska Medical Center in collaboration with Lynx Therapeutics. In this trial which involved 5 dose groups of 3 patients each who received up to 4.5 g over 10 days for a body weight of 75 kg, no remarkable toxicities were found.

Oncogene-targeted oligodeoxynucleotides might also be utilized in combination with conventional purging agents under conditions that favor the killing of malignant cells and the sparing of a high number of normal progenitor cells. To this end, the bone marrow purging drug, mafosfamide, was utilized at low doses in combination with bcr-abl antisense oligodeoxynucleotides, to eradicate $Philadelphia^1$ cells from a mixture of normal and leukemic cells. Full eradication of leukemic cells and the sparing of a significant number of normal progenitors was demonstrated by *in vitro* clonogenic assays and reconstitution experiments in immunodeficient mice (36).

5. Pharmacokinetics and biodistribution of phosphorothioate oligodeoxynucleotides in animals

The *in vivo* efficacy (and toxicity) of any antisense agent is controlled not only by its ability to interact with a biologically relevant target, but also by its pharmacokinetics and biodistribution, which refer, respectively, to the time course of appearance in plasma and urine, and the percentage of dose found in an organ at a given time.

Distribution analysis of bcr-abl antisense oligodeoxynucleotides in mouse tissues by DNA hybridization with a ^{32}P-labeled oligomer complementary to

the injected oligodeoxynucleotide revealed interacting antisense oligodeoxynucleotides throughout the body, but accumulation in the liver 24 h and 72 h after the last oligodeoxynucleotide injection (12). Intact oligodeoxynucleotides were detected in the kidney and liver up to 14 days after the last injection. Accumulation of the bcr-abl phosphorothioate oligodeoxynucleotide in various organs was also assessed by measuring the amount of ^{35}S-labeled material in weighed organ samples; tissue concentrations correlated with the relative levels of intact oligodeoxynucleotides detected in the same tissues and ranged from 3 to 26 mM (not shown). Because phosphorothioate oligodeoxynucleotides undergo relatively slow degradation in mice tissues (37), the 9-day treatment schedule in SCID mouse appeared to reach tissue concentrations in every tissue except brain that would be sufficient to inhibit the growth of primary leukemic cells while sparing the normal cells (36,38).

Based on these data and the availability of sufficient amount of compound, potential therapeutic concentrations of antisense phosphorothioate in plasma and various organs of patients can be reached by continuous i.v. infusion. Analogous to the treatment of colorectal cancer patients with, for example, 5-fluorouracil, antisense [S]ODNs can most likely be administered with a portable, external, infusion-device connected to a subcutaneously implanted venous catheter, thus allowing for outpatient treatment.

6. Conclusion

The rationale for using antisense oligonucleotides as oncogene-targeted therapeutic agents in human leukemias requires the demonstration that specific gene abnormalities have a role in maintaining the leukemic phenotype, and that synthetic oligonucleotides preferentially or selectively affect the survival of leukemic cells. The results of pre-clinical investigations to assess the therapeutic potential of antisense oligodeoxynucleotides as *ex vivo* and *in vivo* anti-leukemia agents have been encouraging, probably beyond the expectations of only a few years ago.

It must also be emphasized that though simple in theory and execution, antisense experiments may be far more difficult to conduct and interpret than the above discussion would indicate. The reasons for this are multifactorial, but relate to an often frustrating inability to identify a suitable region of an mRNA to target, or to non-sequence dependent effects on cell function. These problems, in turn, are likely to be related to mRNA secondary and tertiary structure, and the particular chemistry of the oligo employed, respectively. This does not mean, however, that carefully controlled experiments cannot be informative. Rather, the caveat here is not to confuse a biological effect with an "antisense effect", though the so-called non-specific effects could obviously be advantageous if appropriately exploited.

The application of such compounds as therapeutic agents is still at the stage of infancy. New ODN chemistries are being developed, as are potentially novel methods for their delivery to cells. Only time, a great deal of effort,

and considerable patience will tell whether such compounds are the much longed for "magical bullet" for the treatment of human malignancies (39,40). We must also admit the possibility that they may ultimately be found to be useful only as adjuvants to more conventional therapy, or, less likely, that they may play no role at all in the treatment of human malignancies. At the present time, however, the antisense approach links biology and medicine in the exciting and, hopefully, realistic promise of "rational therapy" for human malignancies.

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Summary

In recent years, the antisense strategy for cancer therapy has progressed from *in vitro* culture studies, to investigations in animal models, and now to clinical studies. We describe here the current state of progress toward gene-directed antisense-based cancer therapy targeting BCR/ABL oncogene, primarily from the viewpoint of initial proof-of-concepts studies in animal models of human leukemias and phase I clinical investigations.

Key words:
antisense strategy, cancer therapy, BCR/ABL oncogene, human leukemias.

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