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ANTISENSE OLIGONUCLEOTIDES: Promise and Reality

Irina Lebedeva and CA Stein
College of Physicians and Surgeons, Columbia University, New York, New York 10032; e-mail: stein@cuccfa.ccc.columbia.edu

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■ **Abstract** Antisense oligonucleotides have been used for more than a decade to downregulate gene expression. Phosphodiester oligonucleotides are nuclease sensitive, and the more nuclease-resistant phosphorothioate oligonucleotides are now in common use in the laboratory and have entered clinical trials. However, these molecules are highly bioactive and may inhibit gene expression by more than one mechanism. Although some dramatic successes have been demonstrated, it can still be difficult to properly interpret experimental data derived from the use of this class of oligonucleotide. This review discusses some of these issues with particular reference to a major area of current interest—inhibition of *bcl-2* expression in tumor cells.

INTRODUCTION

Antisense biotechnology is a potentially powerful tool for the generation of specific gene knockouts. However, it could be quite legitimately questioned whether this promise has attained levels commensurate with the extraordinary reception that greeted its initial successes more than two decades ago. There can be little doubt that, although the antisense approach is a conceptually elegant strategy, formidable difficulties have emerged in its application. In this review, many of the problems that have led to difficulties in interpretation of data obtained from antisense experiments are discussed. This is done with no asperity or sense of doomsday nihilism but in the firm belief that only by dealing with the difficulties in a systematic, scientific, and open manner can genuine progress be achieved.

Ideally, the knockout of a specific gene could be accomplished by taking advantage of knowledge of a portion of the unique sequence of the messenger RNA (mRNA) transcribed from that gene. The synthesis of a small oligonucleotide that is complementary to that sequence would, in theory, take advantage of the exquisite specificity of the Watson-Crick base-pair interaction to inhibit the translation of that gene into protein. If the protein product of translation were important for cell growth and/or viability, then antisense inhibition of gene expression could create a specific knockout phenotype. Indeed, the specificity of the Watson-Crick

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base-pair interaction is such that a specific 15- to 17-mer sequence has been estimated to occur only once in the entire human genome (1).

Although it is relatively easy to synthesize phosphodiester oligonucleotides, they cannot be used as antisense reagents, because they are too readily digested by nucleases. Nucleases are virtually ubiquitous, being found in plasma and intracellularly as well. Digestion of a phosphodiester oligonucleotide appears to occur primarily by $3' \rightarrow 5'$-exonucleolytic activity (2), but some evidence exists for endonucleolytic digestion as well. Equally unfortuitously, the nucleoside monophosphate digestion products can be toxic (3).

To eliminate problems resulting from nuclease sensitivity, modifications have been made to the oligonucleotide backbone. One of the earliest such modifications was replacement of one of the nonbridging oxygen atoms at each phosphorus in the oligodeoxynucleotide chain by a methyl group, producing a methylphosphonate oligodeoxynucleotide (4, 5). This substitution renders the oligonucleotide nuclease resistant, but unfortunately it also produces other changes in the chemical nature of the molecule that reduce its antisense activity. One such change is that methylphosphonate oligonucleotides, in contrast to the isosequential phosphodiesters, are uncharged.

Relative Lack of Activity of Uncharged Antisense Oligonucleotides

With the elimination of negative charge in the methylphosphonate oligonucleotide, aqueous solubility is dramatically reduced, leading to formulation difficulties. Furthermore, although it was not appreciated at the time, loss of charge eliminates several other key properties of antisense oligonucleotides that play a major role in their activity [e.g. loss of RNase H competency (see below; 6)]. This loss of activity is not confined to methylphosphonates; there are in fact no uncharged species of antisense oligonucleotides that have much activity in experiments in tissue culture in the absence of heroic delivery methods, such as the scrape-loading technique that is required for activity with morpholino-oligonucleotides (7). Other oligonucleotides with little intracellular activity include peptide-nucleic acids, whose polypeptide backbones are also uncharged. Although it was originally thought that uncharged oligonucleotides could passively diffuse across cell membranes, it is now recognized not only that they are too large but that the presence of the nucleobases renders these oligonucleotides far too polar for passive diffusion (8). Rather, oligonucleotides, charged or uncharged, enter cells essentially via two processes—adsorptive endocytosis and fluid-phase endocytosis (pinocytosis) (9).

At lower concentrations, the adsorptive component of internalization is undoubtedly much greater than the fluid-phase component. However, it appears that adsorption to the cell surface depends predominately on charge; hence, phosphodiester and phosphorothioate oligonucleotides adsorb well to the cell surface and are internalized well. The opposite is true for methylphosphonates, peptide nucleic acids, morpholino-oligomers, and all other forms of uncharged oligonucleotides.

In addition, there is another major reason why uncharged oligonucleotides are relatively inactive intracellularly—they cannot elicit RNase H activity, which is elaborated on below, after the discussion of phosphorothioate oligonucleotides (6).

Phosphorothioate Oligodeoxynucleotides

The next major backbone modification made to improve nuclease stability was the substitution of a sulfur atom for a nonbridging oxygen at each phosphorus, producing a phosphorothioate oligodeoxynucleotide (10). These molecules are nuclease-resistant, but they are not nuclease proof. More correctly (because, when machine synthesized, each phosphorothioate linkage is a mixture of optically active Rp and Sp diastereomers), it is the Sp diastereomer that is relatively nuclease resistant. The Rp diastereomer is actually equally nuclease sensitive as a phosphodiester linkage (11). Unfortunately, the oligonucleotide that contains all-Rp diastereomers also forms a high-melting-temperature hybrid with a target mRNA (12).

Mixed diastereomeric phosphorothioate oligonucleotides will hybridize to their target mRNAs, but the T_m is significantly lower than that of the isosequential phosphodiester oligonucleotide (13). Whereas each GC and AT phosphodiester pair contributes $\sim 4^\circ$C and 2°C, respectively, to the T_m, each isobase phosphorothioate contributes only $\sim 2.5^\circ$C and 1.5°C, for GC and AT, respectively. The retention of charge, however, retains the property of aqueous solubility, and phosphorothioate oligonucleotides adsorb well to the surfaces of diverse cell types (9). Phosphorothioates have been used successfully in a large number of experiments designed to downregulate gene expression, and they have been and are currently used in several clinical therapeutic trials. Some of the many targets whose expression has been significantly, reproducibly, and convincingly downregulated include protein kinase C-α [PKC-α (14, 15)], c-raf kinase (16), Ha-ras (17), intercellular adhesion molecule 1 (ICAM-1) (18), bcl-2 (19–22), and bcl-xL (23–25). However, despite these and other successes, the interpretation of data derived from the use of phosphorothioate oligonucleotides is complicated. These molecules are highly biologically active, and it is often relatively easy to mistake artifact for antisense. Some of the reasons for this are discussed below. In addition, sets of recommendations have slowly been accumulating that are designed to improve and specify the types of control sequences that should be used in all antisense experiments, especially those with phosphorothioates. Before commencing any experiment with phosphorothioate oligonucleotides, it is advisable to consult these recommendations, which represent the distillation of many years of experience (26).

Nonspecific Effects of Phosphorothioate Oligonucleotides

Any discussion of phosphorothioate oligonucleotides must occur not only in the context of the specificity of Watson-Crick base pairing but also in that of their nonsequence-specific interactions as well. The substitution of sulfur for oxygen not only improves nuclease resistance but creates a molecule of very different

properties from its isosequential parent. One of the major problems with phosphorothioate oligonucleotides is their ability to bind in a length- and somewhat sequence-dependent manner to heparin-binding proteins (27, 28). In fact, other polyanions such as suramin and pentosan polysulfate have long been known to bind to heparin-binding proteins, although they bind with far lower affinity than phosphorothioates. These proteins include basic fibroblast growth factor (bFGF) and acidic fibroblast growth factor, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) (27, 28) and its receptors, and the epidermal growth factor (EGF) receptor (EGFR) but not EGF (29). For bFGF, the binding can be of low nanomolar affinity and can remove bFGF from its low-affinity binding sites in the extracellular matrix (27). For EGFR, phosphorothioate oligomers can induce autophosphorylation of the EGFR in the absence of EGF and will block the binding of EGF to its receptor (29). Phosphorothioate oligodeoxynucleotides can also bind to a wide variety of other heparin-binding proteins, some of which, including fibronectin, laminin (30), and Mac-1 (CD11-CD18) (31), have cellular adhesive properties. In addition, these oligodeoxynucleotides can also bind to CD4 (32), human immunodeficiency virus glycoprotein 120 (33), the human immunodeficiency virus type 1 reverse transcriptase (34), certain isoforms of PKC (35), and RNase H (36). Under certain circumstances, phosphorothioate oligonucleotides can also activate the Sp1 nuclear transcriptional regulatory factor, an effect similar to that of suramin (37).

A striking example of the nonspecific interaction of phosphorothioate oligonucleotides with thrombin protein was provided by Bock et al (38), who enriched a random population of oligonucleotides and isolated the consensus sequence 5′-GGTTGGTGTGGTTGG-3′. This oligonucleotide was a potent inhibitor of the thrombin-catalyzed conversion of fibrinogen to fibrin. However, this molecule does not exist as a randomly coiled single strand; because of the presence of multiple GG dimers, it can fold on itself to form an intramolecular tetraplex. Other intramolecular tetraplexes have been shown to bind with high affinity to molecules such as reverse transcriptase (39).

Phosphorothioate oligonucleotides with four contiguous guanosine residues can form quadruple-stranded tetraplexes and other higher-order structures (40, 41). These highly negatively charged molecules can be extremely biologically active, but their activity may have little if anything to do with the potential antisense properties of the single strand. Unfortunately, it is difficult to know a priori which single-strand sequences have the capacity to form tetraplexes or other higher-order structures under physiologic conditions. For example, Gewirtz and Calabretta had studied an 18-mer antisense *c-myb* phosphorothioate oligonucleotide containing four contiguous guanosine residues (42). This molecule downregulated c-*myb* expression in hematopoietic tumor cells both in tissue culture and in xenografted tumor models. Furthermore, clinical trials in patients with chronic myelogenous leukemia are currently ongoing. It was believed (43), based on evidence from slab gels, that this molecule did not form higher-order structures (e.g. tetraplexes), but

additional evidence from capillary gel electrophoresis, circular-dichroism analysis, and fluorescence spectroscopy demonstrated the presence of higher-order structures in solution (M Vilenchik, L Benimetskaya, P Miller, CA Stein, unpublished data). It is probably the case that, the longer the oligonucleotides and the more centrally located the four contiguous guanosine residues are in the molecule, less is the likelihood of higher-order-structure formation. Notwithstanding these effects, when selecting antisense oligonucleotides for antisense experiments, the consensus opinion is to avoid the problem entirely by discarding oligonucleotides with four contiguous guanosine residues.

Selection of Active Antisense Oligonucleotides

It was originally thought that antisense oligonucleotides should be targeted to an "open" region, e.g. the initiation codon region, of the mRNA. Subsequent experience has shown that this is neither a reliable nor productive target site. In fact, Mir & Southern (44) have suggested that optimum target sites contain a small single-stranded region at which nucleation takes place, plus a double-stranded, helically ordered stem that is invaded by the antisense molecule with displacement of one of the strands. This largely eliminates the unfavorable energetics consequent to reforming a double helix from what essentially would be two single strands. However, selection of active antisense oligonucleotides still remains a somewhat trial-and-error process, although more recently Patzel et al (45) have developed a very promising "in silico" approach to the selection of active sequence. Until this methodology is more generally available, it is believed best to screen 20–40 oligonucleotides, generated randomly by mRNA "walking," to discover active moieties. Oligonucleotides should always be delivered with a carrier (usually a cationic lipid), and the concentrations and molar ratios of carrier and oligonucleotide must be optimized (see 46 for suggestions on the ratio of carrier to oligonucleotide). This is an expensive and laborious process, especially in light of the general consensus, based on large numbers of experiments, that, for every approximately eight oligomers tested against any particular mRNA, only one will be active. The observations of Tu et al (47) bear particular relevance to this problem. These authors accumulated 2026 citations of "successful" antisense inhibition from the literature. In 1655 citations (82% of the total), only one antisense oligomer was evaluated. In 248 (12.2%), two or three of these oligomers were tested, and in 81 (3.9%) four to nine oligomers were tested. Only 42 (2.1%) reports examined >10 oligonucleotides. This information does not jibe with the one in eight (12.5%) activity shown in a number of careful studies. As suggested previously, it is possible that the published literature represents a selection bias and that actually up to eightfold more unique oligomers than the 1655 (i.e. $\leq$13,240) have been evaluated, with the literature reporting only the 12.5% positive results. This appears unlikely, however, because certain sequence motifs have been shown to be highly over-represented in a group of "successful" experiments (48).

Therefore, it is possible that may of the 1655 citations in which only one oligomer was tested do not represent an "antisense" observation but instead combinations of antisense plus nonsequence specificity or perhaps antisense plus cytotoxicity. Regardless, it is difficult to determine whether this is the case unless extensive, well-controlled experimentation is performed. Unfortunately, for various reasons not all of which are scientific, such studies have rarely, if ever been undertaken.

RNase H and the Problem of "Irrelevant Cleavage"

The antisense effect is probably mediated by RNase H (49, 50), an enzyme that cleaves the mRNA strand at the site of hybridization of a complementary strand of DNA. This has been demonstrated in a variety of systems, including wheat germ extract (51) and *Xenopus* oocytes (52); RNase H activity also probably mediates the antisense effect in intact mammalian cells, as shown by the work of Giles et al (53). These investigators were able to detect RNase H-generated 3′ fragments of the p53 mRNA after microinjection of an antisense p53 oligonucleotide.

Backbone chemistry determines RNase H competency. Competent backbones have phosphodiester and phosphorothioate linkages (13), and the sugar moiety must be deoxyribose. Molecules containing uncharged backbones, composed for example of methylphosphonate (54) or peptide nucleic acid linkages, and molecules that exclusively contain either N3′-P5′ phosphoramidate linkages or 2′-*O*-methylribose (or any substitution at the 2′ position) are not competent. RNase H competence appears to play a critical role in the production of antisense activity. If the backbone of an active molecule is modified so that it is no longer RNase H competent, antisense activity may be totally lost.

Most critically, RNase H is an enzyme of rather low "stringency"; it does not require a fully complementary Watson-Crick base-pair hybrid between oligonucleotide and mRNA for elicitation of its activity. Cazenave et al (51) worked with wheat germ extract and observed that RNase H could cleave β-globin mRNA at a site that was only partially complementary (13 base pairs) to a 17-mer antisense oligonucleotide. Indeed, Monia et al (55) have demonstrated that very short oligonucleotides consisting of only a five- (and perhaps even as low as four-) contiguous-base region of homology may, under some circumstances, be sufficient to elicit RNase H activity. The appreciation of the low "stringency" of RNase H has also led to the counterintuitive proposal (56) that oligonucleotide specificity is actually inversely related to its length, that is, that the longer the length of an RNase-H competent oligonucleotide, the less specific it becomes. This diminution in specificity is directly attributable to the fact that, as the length of the oligomer increases, so does the number of nested short sequences. For example (56, 57), any 15-mer can be viewed as containing eight overlapping 8-mers. However, each 8-mer represents a different potential RNase H cleavage site. Moreover, each site would then be expected to occur approximately once in every 6.55×10^4 bases of random sequence or once in every 8192 bases for the sum of the eight

sequences. This is much greater than the 1 in 10^9 sequences predicted purely on the basis of 15-mer sequence homology considerations. However, it is also likely that few of these 8192 sites are available for hybridization or accessible to RNase H. Nucleation and zippering, two initial events in duplex formation, can probably occur only in select regions of defined mRNA structure (44).

Nontargeted mRNA cleavage by RNase H has been informally termed "irrelevant cleavage." The extent to which it occurs probably depends not only on oligomer length but also on backbone chemistry and concentration. The intracellular oligonucleotide concentration, in turn, may depend on the nature of the agent used to deliver it.

Irrelevant Cleavage May Occur in Intact Cells

Treatment of T24 human bladder carcinoma cells with a 20-mer all-phosphorothioate (15, 58) oligodeoxyribonucleotide (Isis 3521), targeted to the 3′-untranslated region of the PKC-α mRNA, led to almost total downregulation of PKC-α protein and mRNA. The oligonucleotide was delivered as a complex with Lipofectin™ (GIBCO BRL; Rockville, MD). The authors of this report claimed that other isoforms of PKC (β1 and ζ), were not inhibited.

However, in other experiments, when Isis 3521 was delivered in complex with the cationic porphyrin tetra-*meso* (methylpyridyl) porphine, inhibition was both of PKC-α protein and mRNA expression and of PKC-ζ (although not of PKC-β1, δ or ε) protein and mRNA expression (59). In addition, the expression of the 4.0- and 2.2-kb PKC-ζ transcripts was reduced by $>$95%, whereas levels of control PKC-λ/ι mRNA were not markedly changed. In contrast to the original report, identical co-downregulation of PKC-α and PKC-ζ was observed also when Lipofectin™ was used as the carrier. This co-downregulation may be caused by irrelevant cleavage. There is a contiguous 11-base match between Isis 3521 and the PKC-ζ mRNA, which, in theory and certainly in cell-free systems, is more than sufficient for RNase H competency.

Isis 3522, a 20-mer phosphorothioate oligonucleotide which is targeted to the 5′ region of the PKC-α mRNA, inhibits PKC-α protein and mRNA expression, but does not inhibit PKC-ζ expression. Here, only a 4-base region of complementarity exists between the oligonucleotide and the PKC-ζ target. Such a small region of homology is probably insufficient for RNase H competency, and so irrelevant cleavage does not occur. However, the relative ease with which potential irrelevant cleavage was found in this system suggests that it is a far more common problem than usually contemplated. If so, then it may be very difficult to relate an observed phenotype to a specific knockout, precisely because the knockout is not as specific as assumed.

Potential Elimination of Irrelevant Cleavage

Conceptually, this is straightforward; if RNase H activity is eliminated, then the potential for irrelevant cleavage is also eliminated. However, the elimination of

RNase H competency unfortunately creates additional problems. A partial decrease in the RNase H competency of an oligonucleotide will occur if the backbone charge is reduced. However, if charge reduction is excessive, insolubility and formulation difficulties will be the result. Furthermore, the ability of cationic carriers to deliver the oligonucleotide will be drastically diminished, and antisense efficacy will be compromised. Uncharged molecules are usually not very active antisense effectors, because they must depend on steric blockade of translation for efficacy. This is usually ineffective because the 80S elongating ribosomes have intrinsic unwinding ability (60, 61), and they can probably read through the steric block.

Charge may be retained by substituting nuclease-resistant but non-RNAse H-competent 2′-*O*-alkyloligoribonucleotides throughout the length of the backbone. This retains the property of aqueous solubility, but efficacy will most likely be greatly diminished, probably also because of ribosome-promoted unwinding. Perhaps the most effective strategy yet devised to reduce yet not eliminate RNase H competency is the use of so-called "gap-mers," which have six to eight 2′-*O*-alkylloligoribonucleotides (alkyl = methyl or methoxyethoxy) at the 3′ and 5′ termini and, to retain RNase H activity, a central core of six to eight oligodeoxyribonucleotides. For purposes of nuclease resistance, the entire backbone contains phosphorothioate linkages. These oligonucleotides tend to be somewhat expensive to synthesize, and, although extremely promising, their use is not yet as widespread as perhaps it should be.

Another strategy for eliminating irrelevant cleavage calls for the replacement of RNase H competency by RNase P competency [see Altman (62) and Forster & Altman (63)]. RNase P, like RNase H, is a ubiquitous cellular enzyme, functioning to cleave the 5′ terminus of precursor transfer RNA (tRNA) molecules to generate a mature tRNA. If a synthetic oligonucleotide [called an external guide sequence (EGS)] targeted to an mRNA is designed to mimic certain structural features of precursor tRNA (i.e. to incorporate a stem and seven-residue loop hairpin, in addition to the two hybridizing arms linked to the 3′ and 5′ ends of the stem loop), then in cell-free systems RNase P will cleave the target RNA at the junction between the single-stranded leader sequence and the duplex formed with the EGS (64).

Ma et al (64) have recently developed a series of nuclease-resistant, serum-stable EGSs that efficiently induce RNase P cleavage in vitro of a 29-mer derived from the hepatitis B virus genome. More recently we have used an EGS whose sequence was extrapolated from Isis 3521 to downregulate PKC-α expression in T24 bladder carcinoma cells. Two carriers were used, Lipofectin^{TM} and LipofectACE^{TM} (GIBCO BRL; Rockville, MD), with identical results—almost 95% downregulation of protein and mRNA expression. In contrast to what we observed with phosphorothioate oligonucleotides, this occurred in the absence of any downregulation of PKC-ζ mRNA or protein expression. Excellent downregulation of bcl-xL protein and mRNA expression in T24 cells was also demonstrated by the use of the EGS technology. However, EGSs are presently difficult and expensive to synthesize.

Antisense Oligonucleotides Targeted to bcl-2: Tissue Culture Successes, Problems of Data Interpretation, and Initiation of Clinical Trials

Bcl-2 is an important antiapoptotic protein found in a wide variety of human cancer cells. Its inhibition would theoretically sensitize cells to cytotoxic chemotherapy, and a number of at least partially successful studies in tissue culture and experimental animals have been performed that have led to the initiation of clinical trials.

Initially, antisense phosphodiester and phosphorothioate oligonucleotides targeted to the bcl-2 mRNA were used to inhibit the growth in culture of 697 human leukemia cells (65). The oligonucleotide targeted the translation initiation site of the human bcl-2 mRNA. Both classes of oligomer decreased cell proliferation: The phosphorothioates were more potent inhibitors, but, given the benefit of a decade of research, the experiments with the phosphodiesters should now probably be discounted. It was proposed the phosphorothioate bcl-2 antisense oligonucleotides induced cell death through sequence-specific mechanisms, but in retrospect this is difficult to accept because they were not delivered to the cells by a carrier and the concentrations were high (25 μM). These problems (i.e. lack of carrier delivery, high concentration, and use of only a single control) recur in almost all of the reports that target bcl-2 except for a few that are specifically noted below. Furthermore, this oligonucleotide was empirically chosen to target the translation initiation site of the bcl-2 mRNA, a method no longer considered to be an appropriate one for identifying active oligonucleotides.

In other work with the phosphorothioate oligonucleotide used by Reed et al (65), Bcl-2 protein expression was inhibited in acute myeloid leukemia cells. This was associated with decreased duration of cell survival and a diminution in the number of clonogenic cells in culture (66). Subsequent to bcl-2 downregulation, treatment with daunorubicin and 1-β-D-arabino-furanosyl-cytosine (AraC) increased cell kill (66, 67). Durrieu et al (67) used the same oligonucleotide as Reed et al (65), but the concentration was lower (1 μM) because delivery was accomplished with cationic lipids. However, only a single control oligomer was used. In another set of experiments, in 7 of 17 samples of myeloblasts from acute myeloid leukemia patients, treatment with the antisense oligonucleotide resulted in a significant decrease in the expression of Bcl-2 protein (68). This, in turn, was accompanied by increased apoptosis in response to AraC. These experiments were more rigorously controlled than those of Reed et al (65), and the oligonucleotide concentration was lower (5 μM). However, no carrier was used for delivery, introducing questions of specificity.

Phosphodiester and phosphorothioate antisense oligonucleotides directed against the first six codons of the Bcl-2 mRNA (denoted G3139; Genta, Inc., Lexington, Mass.) were also successfully used in non-Hodgkin lymphoma cell lines. A putatively specific reduction in Bcl-2 mRNA levels within 1 day of treatment was demonstrated by Kitada et al (69), in SU-DHL-4 cells. A commensurate

reduction in Bcl-2 protein level occurred only after 3 days, presumably because of the long half-life of the Bcl-2 protein, and this reduction was associated with a drastic decrease in cellular viability. Treatment of RS11846 lymphoma cells with G3139 similarly reduced Bcl-2 expression and sensitized these cells to AraC and methotrexate (70). However, the oligonucleotide concentration was extreme (200 μM), no carrier was used for delivery, and the authors of that report used only a single control sequence.

Overexpression of Bcl-2 has also been observed in human prostate cancers (71). Although Bcl-2 expression in normal prostatic epithelial cells is low or absent, Bcl-2 is ultimately upregulated in $\sim$35% of the tumors from patients after progression to androgen independence (72). The forced overexpression of Bcl-2 protein in LNCaP prostate cancer cells increased their in vivo tumorigenic potential and resistance to apoptosis (73).

Treatment of LNCaP (74) and Shionogi tumor cells (75) in vitro with G3139 inhibited Bcl-2 expression via a dose-dependent and sequence-specific manner. The authors used the cationic lipid Lipofectin^{TM} for delivery, and measured Bcl-2 protein and mRNA levels. Unfortunately, they used only a single control oligonucleotide, which diminishes the credibility of the experiments. In other experiments, antisense *bcl-2* oligonucleotide treatment substantially enhanced paclitaxel and docetaxel chemosensitivity in a dose-dependent manner. However, characteristic apoptotic changes were demonstrated only after combined treatment (22, 76) and not after treatment with the Bcl-2 oligomer alone.

Miyake et al have tested the efficacy of G3139, administered as an adjuvant after castration, to delay the time to androgen-independent recurrence in the androgen-dependent mouse Shionogi tumor model (22, 74–76). Systemic administration of G3139 beginning 1 day postcastration in mice bearing the Shionogi (75) or LNCaP (74) tumors resulted in a more rapid regression of tumors and a significant delay in the emergence of androgen-independent recurrent tumors than in animals not exposed to the oligonucleotide. In addition, despite significant reductions of Bcl-2 expression in tumor tissues, G3139 had no effect on Bcl-2 expression in normal mouse organs (75). Adjuvant in vivo administration of G3139 and micellar paclitaxel after castration resulted in a statistically significant delay in the appearance of androgen-independent, recurrent tumors compared with administration of either agent alone (22). When this regimen was given to mice bearing established Shionogi tumors, tumor regression was far more dramatic when compared with treatment with either agent alone (76). These findings illustrated the potential utility of an antisense bcl-2 approach for prostate cancer in the adjuvant setting when combined with androgen ablation and taxane treatment.

Antisense *bcl-2* Oligodeoxynucleotides In Vivo

Phosphorothioate oligonucleotides (including G3139) have been infused intravenously or intraperitoneally into patients taking part in clinical trials, and their pharmacokinetics have been extensively studied (77, 78). These oligonucleotides

are stable for 48 h (15–50%), at which time levels may still be detected in tissues. Plasma clearance is biphasic, with half-lives on the order of 15–25 min and 20–40 h. For G3139, after an intravenous bolus dose of ∼5 mg/kg, the plasma elimination half-life is 22 h (79).

Cotter et al (80) obtained cells from a patient with B-cell lymphoma bearing the t(from 14 to 18) translocation and, after treatment with G3139 in tissue culture, demonstrated downregulation of Bcl-2 protein and induction of apoptosis. They then xenografted these cells into a severe combined immunodeficient (SCID) mouse model (80). Preinoculation treatment of the cells with G3139 ablated the ability of the cells to grow in the host. Congruently, G3139 almost completely abolished lymphoma growth in 50 of 60 treated mice (83%) after 2 weeks. Extension of treatment to 3 weeks completely eradicated lymphoma in all animals, even at the PCR level (81). The effect appeared specific, but one confounding fact is that G3139 contains two CpsG sequences, which can activate residual natural killer (NK)-cell activation (82). However, the experiments also were repeated in NOD/SCID mice which lack NK-, B-, or T-cell activity, and similar efficacy was observed (83).

A phase I study in non-Hodgkin lymphoma patients with high Bcl-2 expression (84, 85) was then undertaken. No treatment-related toxicities were observed at doses of 5 mg/kg/day (84). One patient remained in remission for 3 years after starting treatment in the absence of additional therapy. No other patients achieved a complete response. In two patients, computer tomography scans demonstrated some reduction in tumor size (84), but partial response status was not achieved. Nine patients had disease stabilization and at least two of them had symptomatic improvement. Nine additional patients had progressive disease (85). To use a surrogate pharmacodynamic measurement, levels of Bcl-2 protein were measured by flow cytometry in peripheral blood samples, and these levels were found to be reduced in 7 of 16 assessable patients. Additional trials in this disease are currently in progress. G3139 also appears at this time to be able to chemosensitize melanoma cells. Treatment of melanoma cells in vitro with G3139 (200 nM) in complex with a cationic lipid led to what was proposed to be a sequence-specific and dose-dependent downregulation of the Bcl-2 mRNA (20). Two control oligonucleotides were ineffective. Chemosensitization of human melanoma cells also occurred after treatment of melanoma xenografts with G3139 in SCID mice, and a combination of G3139 and dacarbazine resulted in complete ablation of the tumor in three of six animals. In a currently accruing phase I–II study, dacarbazine and G3139 are being combined in patients with advanced disease, including patients resistant to prior single-agent dacarbazine (86). Initial results from this study indicate that G3139 can reduce Bcl-2 expression in the xenografted melanoma deposits and that combined therapy with dacarbazine is well tolerated. G3139 in combination with docetaxel is also in phase I trials for patients with advanced breast cancer and other solid tumors, and at this point the regimen's toxicity has been tolerable (87). Tumor response was observed in two patients with breast cancer.

The safety data from clinical trials support further clinical development of G3139, both as a single agent and in combination with cytotoxic agents. G3139 now is also in phase I/IIA trial for patients with androgen-independent prostate cancer, in which setting the G3139 will also be combined with docetaxel. Trials in other advanced solid-tumor malignancies have been planned (88). The relative clinical success with G3139 to date is tremendously encouraging: Because its toxicity appears to be relatively low, consisting mainly of fatigue and thrombocytopenia, this molecule may represent a truly novel advance in the treatment of human solid tumors, irrespective of its mechanism of action, which may or may not be related to inhibition of bcl-2 expression. However, from the point of view of the patient whose tumor may be responding to therapy, questions of mechanism are wholly moot points.

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