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Review article

Potential roles of antisense oligonucleotides in cancer therapy. The example of Bcl-2 antisense oligonucleotides

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Abstract

Antisense oligonucleotides have been widely used to specifically and selectively downregulate gene expression at the messenger RNA level. Even though oligonucleotides are commonly used in laboratories and clinical trials, they can induce non-specific effects that can lead to misinterpretation of experimentally-derived results. This review summarizes precautions one should take when using oligonucleotides. In addition, the role of one oligonucleotide, G3139, which is targeted to the coding region of bcl-2 messenger RNA, in inhibiting tumor progression in vitro and in clinical trials, is described.

Keywords: Antisense oligonucleotide; Bcl-2; G3139; Human trials

1. Introduction

The principles that underline antisense biotechnology are conceptually simple and elegant. Oligonucleotides, short sequences of DNA, have been designed to hybridize by virtue of the specificity at the Watson–Crick base-pair interaction to a defined target messenger RNA and can inhibit its translation into protein. This approach was first employed in 1978 by Zamecnik and Stephenson to inhibit the Rous sarcoma virus expression in chicken fibroblasts, and has subsequently been greatly elaborated [1]. To date, numerous oligonucleotides are now in clinical trials [2,3]. One has recently been commercialized (Vitravene for cytomegalovirus retinitis).

2. Antisense oligonucleotide: use and limitations

Even though the antisense strategy is widely employed currently, it has certain defined limitations. Although it is relatively easy to synthesize phosphodiester oligonucleotides, these cannot be used as drugs due to their propensity to be easily degraded by cellular nucleases. To improve their stability against nuclease digestion, many different chemical modifications have been evaluated. These modifications include the use of 2′-*O*-methyl, 2′-*O*-(2-methoxyethyl)oligoribonucleotides, α-anomeric or locked nucleic acid (LNA) oligonucleotides, backbone modifications such as the peptide nucleic acids (PNAs) and morpholinophosphorodiamidates, and phosphodiester linkage modifications such as phosphorothioates (PS), methylphosphonates (MP) and phosphoramidates (PN). All these modifications increase the stability of oligonucleotides in biologic systems, but it also appears that they can either alter the oligonucleotide's capacity to hybridize with its targeted RNA, or its cellular internalization. Modifications leading to uncharged oligonucleotides, such as methylphosphonate, peptide nucleic acid (PNA) or morpholino oligonucleotides, lead to a decrease both in aqueous solubility, and in their ability to penetrate into cells [4,5]. Different methods, such as scrape-loading [6], electroporation [7], microinjection [8] or the binding to particular peptides with membrane translocation properties [9] have been developed to overcome these internalization problems. These methods are easily applied in cultured cells, but may or may not be useful in in vivo systems.

Among the different 'second-generation' modifications studied in the past two decades, most are unable to induce ribonuclease H (RNAse H) activity, the principal nuclease that cleaves RNA at the level of the mRNA/oligonucleotide duplex. This problem has been overcome by using chimeric (or 'gapmer') oligonucleotides, which contain non RNase H-dependent nucleotides at their 5′ and 3′ extremities and in their central region phosphodiester or phosphorothioate

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nucleotides that support RNAse H cleavage. These 'gapmer' oligonucleotides are resistant to cellular nuclease degradation and may have potent antisense effects [10–12].

Due to the base complementary of the genetic code, oligonucleotides are, at least in theory, highly specific and selective. However, a profusion of experiments performed in the last two decades indicate that precautions have to be taken when interpreting data, since one can be easily misled by the potential non-specific effects that oligonucleotide can induce. Indeed, a major problem with antisense oligonucleotides, which is especially true of the phosphorothioates, is their capacity to interact, in a sequence- and length-dependent manner, with numerous proteins such as fibronectin [13], basic fibroblast growth factor (bFGF) [14], human immunodeficiency virus type 1 reverse transcriptase [15], and many others, all of which are heparin-binding. To overcome these problems and conclusively demonstrate an antisense effect, numerous control oligonucleotides must be employed.

It has long been known that the antisense effect is mediated by two major mechanisms: RNA-specific cleavage by ribonuclease H (RNase H) at the hybridization site, and steric-blockade of the translational machinery or splicing progression. RNase H, even though its mechanism of action is not completely understood, appears to be the major antisense effector in a variety of systems. Its activity is elicited by competent oligonucleotides, of which phosphorothioates and phosphodiesters are the only species commercially available. However, RNAse H requires only a 5–6 base-pair region of hybridization to recognize and cleave a mRNA–DNA duplex [12]. This weak requirement of homology can lead to the degradation of non-targeted mRNA molecules [16,17] and must be kept in mind if determination of gene function, rather than therapeutic intent, is considered.

Constraints also affect the use of steric-blocking oligonucleotides. These oligonucleotides are non-RNase H dependent and may act via the inhibition of splicing [18], either by disrupting the ribosomes [19] or by physically blocking the initiation or elongation step of translation [20]. However, most of the efficient steric-blocking oligonucleotides are targeted to the 5′-untranslated region or the initiation codon of the messenger RNA. Targeting the coding region appears to be non-productive as the mRNA may be involved in secondary and tertiary structure formation. Such higher order structure may impede the access of potential antisense oligonucleotides. Indeed, in the coding region, it appears that the ribosomal complex can easily unwind the mRNA/oligonucleotide duplex.

Oligonucleotides can also induce immunostimulatory effects if they contain CpG motifs in their sequence. This immunostimulation takes place by the release of specific cytokines (interleukin (IL)-6, IL-12) or other soluble pro-inflammatory proteins, for example the chemokines macrophage inflammatory protein-1β and monocyte chemoattractant protein-1 [21]. Even in SCID mice, the presence of CpG motifs may be immunostimulatory [22]. To overcome this problem, oligonucleotides containing CpG motifs should generally not be employed in vivo. If necessary, however, selective methylation of the cytosine of the CpG, or the replacement of the phosphorothioate linkage by an oligonucleotide backbone that does not support immune stimulation may be useful. These changes have significantly minimized the immunostimulatory effects of CpG phosphorothioates [23,24].

In order to downregulate gene expression, oligonucleotides must penetrate into cells to reach their target. To date, the precise mechanism of the intracellular penetration of oligonucleotides has not been resolved. As numerous reports have shown, naked oligonucleotides are poorly internalized by cells [4,25]. Thus, experiments in tissue culture must be performed with oligonucleotides complexed to transfection vectors (see Ref. [26] for a review). These vectors can be cationic lipids [27,28], which are the most commonly used carrier system. Liposomes [29,30], polylysine [31], dendrimers [32,33], nanoparticles [34] and polyethyleneimine [35,36] have also been employed. Small peptides, some of which can translocate through the plasma membrane, can be coupled to oligonucleotides to enhance their delivery into cells [37–39]. Another approach consists of transiently permeabilizing the plasma membrane to create pores which allow the oligonucleotide to enter the cells [40,41]. Nevertheless, in only a few in vivo experiments, and to our knowledge in no human clinical trials, have vectors been employed to deliver oligonucleotides [42]. Therefore, the question of the use of vectors in these settings remains an open one.

3. Bcl-2 antisense therapy

The specificity of oligonucleotide hybridization makes antisense treatment an attractive approach to selectively modulate the expression of genes involved in diseases such as viral infection, cancers or inflammatory disorders. There is a pressing need to develop selective anticancer agents which target key genes involved in cancer and malignancy, and which do not possess the undesirable side effects of conventional chemotherapy.

Programmed cell death or apoptosis, a common mechanism used by cells to die, is important in development and the maintenance of homeostasis. Disturbing the apoptotic process may contribute to the development of cancer or autoimmune diseases. One of the first mammalian regulators of apoptosis discovered was the Bcl-2 protein. The gene is activated by the t(14;18) chromosomal translocation in B-cell lymphoma [43] and has now been implicated in a wide variety of cancers such as melanoma, breast, prostate and lung carcinomas. Recent tremendous interest has arisen with respect to the identification of Bcl-2-related proteins. These proteins can be subdivided into two opposing biological categories, as they either promote or inhibit cell death and

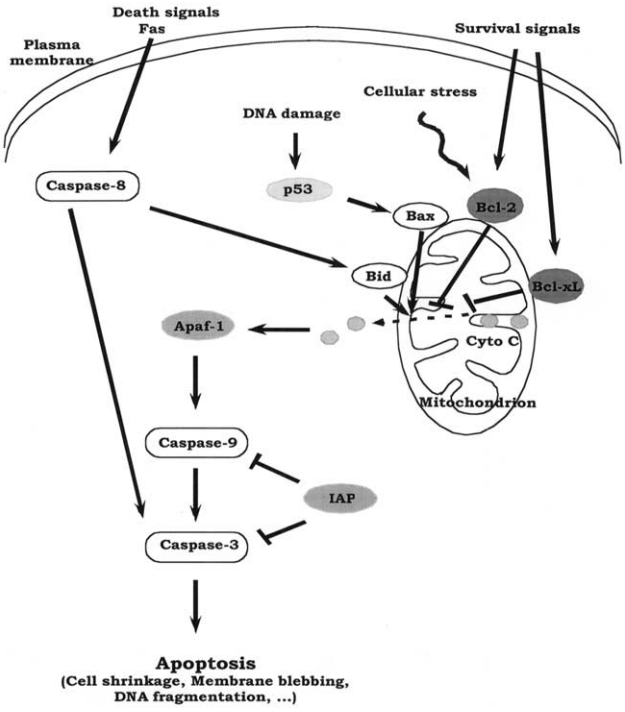

Fig. 1. The different pathways to induce apoptosis. External death signals induce the activation of the caspase pathway, leading to apoptosis. In the intrinsic pathway, the Bcl-2 family proteins translocate to the mitochondria, and induce or inhibit the release of cytochrome *c*, which, once in the cytosol, activates caspase-9 and caspase-3 through the activation of Apaf-1, leading also to apoptosis. These two pathways lead to apoptotic cell death characterized by cell shrinkage, DNA fragmentation and membrane blebbing.

include the anti-apoptotic proteins Bcl-xL, A1, Bcl-w, and Mcl-1 and the pro-apoptotic proteins Bax, Bak, Bok, Bik, Bad, and Bid among others (Fig. 1). All share up to four conserved Bcl-2 homology (BH) domains, which mediate homo- and heterodimer formation, and, for most of them, a hydrophobic C-terminal domain that allows them to be anchored in intracellular membranes such as the mitochondria, endoplasmic reticulum or nuclear envelope [44,45].

Bcl-2 has been implicated not only in neoplasia, but also in resistance to cancer treatment. Vaux et al. [46] showed that overexpressing Bcl-2, by introducing a human bcl-2 gene into lymphoid and myeloid cell lines that require IL-3, increased the viability of the cells even with IL-3 withdrawal and maintained them in a G_0, rather than a proliferating state. Additionally, Reed et al. [47] had demonstrated that stable introduction of a Bcl-2 expression plasmid into Jurkat human T-cell leukemia line induced survival of the cells during serum deprivation. Reed et al. [48] tested the influence of Bcl-2 protein on the sensitivity of lymphoma and leukemia cell lines to cytotoxic drugs commonly used in the treatment of cancer. First, the leukemia cell lines were stably infected with recombinant bcl-2 retroviruses to achieve an increase in Bcl-2 protein expression. This increase correlated with high resistance to the cytotoxic drugs tested in these cells. Secondly, lymphoma cell lines, which have the t(14;18) translocation and express high levels of Bcl-2 protein, were either stably transfected with an inducible bcl-2 antisense expression plasmids or treated with bcl-2 antisense oligonucleotides to achieve reductions in Bcl-2 protein expression. This reduction resulted in enhanced sensitivity to the anticancer drugs. These two experiments indicate that Bcl-2 protein is one of the key determinants of cellular sensitivity to chemotherapeutic drugs. Blocking Bcl-2 protein expression thus appears to be a logical and important target for antisense therapy.

Subsequently, Reed et al. [49] demonstrated that the proliferation of human 697 leukemia cells could be inhibited via an antisense strategy. Two oligonucleotides were tested: a phosphodiester and a phosphorothioate, both targeted to the start codon region of the human bcl-2 mRNA. However, although the bcl-2 expression could be inhibited by this approach, the study was performed without a carrier, and no Northern blot analysis was obtained. In another work, Kitada et al. [50] found that an 18-base phosphodiester oligonucleotide, complementary to the first six codons of the bcl-2 mRNA, completely and selectively abolished Bcl-2 protein expression in a cell free rabbit reticulocyte lysate. A control scrambled oligonucleotide had no effect on Bcl-2 protein expression, and the antisense oligonucleotide also had no effect on chicken bcl-2 mRNA, which contains three mismatches relative to the human bcl-2 mRNA. The same experiment was carried out in a fibroblast cell line that was transfected with a recombinant retrovirus containing the human bcl-2 cDNA, or in a SU-DHL-4 t(14;18)-containing lymphoma cell line. In both cases, a reduction of the level of Bcl-2 expression was observed with the antisense oligonucleotide and not with the scrambled oligonucleotide. Indeed, 84–95% inhibition of Bcl-2 protein expression was obtained for the SU-DHL-4 cells. In the antisense treated SU-DHL-4 cells, complete loss of bcl-2 mRNA was achieved within 1 day, but commensurate reductions in Bcl-2 protein levels did not occur until 3 days, presumably due to the long half-life of the Bcl-2 protein (approximately 14 h). However, here too, the oligonucleotides were added to the SU-DHL-4 cell cultures without any carrier.

In vitro studies led to the identification of G3139, an 18-base phosphorothioate oligonucleotide complementary to the first six codons of the bcl-2 mRNA. This molecule selectively and specifically inhibits Bcl-2 expression in the SU-DHL-4 t(14;18)-containing lymphoma cell line [50], RS11846 lymphoma cells [51] and DoHH2 cells derived from a patient with immunoblastic lymphoma [52]. G3139 has also been evaluated in other tumor cell lines such as prostate cancer cells. In androgen-dependent Shionogi tumor cells [53,54], G3139 inhibited Bcl-2 expression in a dose-dependent and sequence-specific manner. Additionally, the systemic administration of G3139 in mice bearing Shionogi tumors, initiated 1 day after castration, induced a rapid regression of tumors, whereas the oligonucleotide had no effect on Bcl-2 expression in normal mouse organs. The

extent of tumor regression was higher when paclitaxel and antisense were simultaneously administered.

Recently, Zangemeister et al. [55] demonstrated an additional approach to downregulate Bcl-2 protein expression. They showed that a 20-mer 2′-*O*-methyl-ethoxy-modified phosphorothioate oligonucleotide could simultaneously inhibit both Bcl-2 and Bcl-xL protein expression in SW2 lung cancer cells. As these two related proteins have regions of high homology, three oligonucleotides, representing 0, 1, 2 or 3 mismatches with both sequences, were targeted to this homologous region. Even though all these oligonucleotides downregulate mRNA and protein expression, one of them, 4625, which has perfect homology with bcl-2 and 3 mismatches with bcl-xL mRNAs, possessed the strongest antisense activity. This bispecific 4625 oligonucleotide induces cell death, measured by PI uptake analysis. Furthermore, cell death results from apoptosis induction, as an increase of caspase-3-like activity (10- to 100-fold) and nuclear fragmentation and chromatin condensation were demonstrated. Cells treated with a scrambled oligonucleotide, 4626, showed no effect on either bcl-2 or bcl-xL mRNA and protein expression, and no characteristics of cellular apoptosis were detected. The bispecific 4625 oligonucleotide was also tested on the A375 melanoma cell line and a series of primary melanoma cell cultures derived from tumors at different stages (primary tumors, lymph node metastases and distant metastases) [56]. In both cell lines, a decrease of bcl-2 and bcl-xl expression was also demonstrated, which was accompanied by a dose-dependent decrease in cell growth and an increase in cell death and apoptosis. No toxic or cytostatic effects were observed with the scrambled control oligonucleotide 4626. However, the capacity to simultaneously inhibit the expression of two proteins depends on the availability of single-stranded complementary sequences with a high degree of homology between the two mRNAs.

After demonstrating that G3139 downregulated Bcl-2 expression and induced apoptosis in cultured lymphoma cells bearing the t(14;18) translocation, Cotter et al. [52] injected these cells into severe combined immunodeficient (SCID) mice. Lymphoma developed within 28 days. To determine the effect of G3139 on lymphoma development, the cells were first incubated with the oligonucleotide and then inoculated into mice. At 28 days, lymphoma development was specifically inhibited in the antisense treated mice, but the sense and scrambled oligonucleotides were ineffective.

In further experiments using this lymphoma model, Cotter et al. [57] demonstrated that G3139 almost completely abolished lymphoma growth in 50 of 60 treated mice (83%) after a 2-week infusion at a dose of 100 μg/day oligonucleotide. However, the disease was still present in the remaining mice (17%). Extension of the treatment to 3 weeks, at the same dose, completely eradicated the lymphoma in all animals, even at the PCR (polymerase chain reaction) level. The same effect was also observed with a 2-week treatment but with higher doses of oligonucleotide (300 μg/day). No eradication was observed with control oligonucleotide, but the question of specificity still remained as the G3139 oligonucleotide possesses two CpG motifs in its sequence, and is thus capable of NK cell activation. However, methylation of G3139 eliminates immune system activation but does not eliminate the antitumor activity in NOD-SCID mice, which lack functional NK cells.

Because a prerequisite for the elaboration of antisense molecules to clinically active drugs lies in their ability to reach their intracellular targets, phosphorothioate oligonucleotide pharmacokinetics have been extensively studied. In BALB/c mice, G3139, administrated intravenously at 5 mg/ml, was widely and rapidly distributed in many tissues (though mostly to liver and kidney), and was slowly eliminated from the plasma (half-life of 22 h) [58]. Five minutes after administration of a radioactive-labeled oligomer, 98% of the radioactivity was associated with protein. These results are consistent with the high affinity of the phosphorothioate for proteins present in the plasma (e.g. albumin, although there are others) or on the cell membrane.

Given the results obtained in in vitro and in vivo studies, a Phase I trial was performed for lymphoma patients with high Bcl-2 expression, and who also had progressive disease and had failed at least two or more therapeutic treatments. Webb et al. [59] studied the pharmacokinetics and toxicity of G3139 administrated for 2 weeks in doses of between 4.6 and 73.6 mg/m^2 per day, in nine patients with non-Hodgkin lymphoma. Dose-limiting toxicity was not attained. Only one patient achieved a complete response to the antisense treatment. Three had stable disease, whereas the remaining five patients had progressive disease. In two patients, computer tomography scans demonstrated a reduction in tumor size, and one had no lymph-node masses larger than 1 cm. The level of Bcl-2 protein in five of the nine patients was measured by fluorescence-activated cell sorter (FACS) in peripheral-blood cells or in bone marrow or lymph node aspirates samples, and in three patients this level had decreased. Levels of control HLA A, B and C proteins remained unchanged. In another study, to determine the dose-limiting toxicity for G3139, the oligonucleotide was administrated subcutaneously via continuous infusion to 21 patients during 2 weeks, in doses between 4.6 and 195.8 mg/m^2 per day [60]. In this study, no treatment-related toxicities, except for local inflammation at the infusion site for all the patients, were noted below doses of 110.4 mg/m^2 per day dose. After treatment, one patient demonstrated a complete response to treatment, two presented minor responses, nine had stable disease, and the remaining nine patients had progressive disease. Bcl-2 protein, measured by FACS in peripheral blood mononuclear cells or in bone marrow samples or lymph node aspirates in 16 patients, was reduced (>15%) in seven patients, whereas no changes in HLA protein expression were observed. G3139 oligonucleotide was also administered in doses between 0.6 and 6.9 mg/kg per day by 2-week contin-

uous i.v. infusion, in 35 patients with progressive solid tumors, such as prostate cancer [61]. In all these patients, the continuous infusion was well tolerated as the principal side-effect was fatigue (66%), accompanied by low-grade hematologic toxicity. The half-life of G3139 in this administration mode was 2 h, which differs from the 7-h value measured when G3139 was delivered subcutaneously [60]. In one patient, treated for 2 weeks with 4.1 mg/kg per day of G3139, Bcl-2 protein expression was downregulated at day 15 in peripheral blood mononuclear cells.

Because Bcl-2 is also implicated in cellular resistance to chemotherapy or radiotherapy, combinations of the G3139 oligonucleotide with chemotherapeutics drugs have been studied. The in vitro treatment of 518A2 melanoma cells with 200 nM of G3139 oligonucleotide led to a complete loss of bcl-2 mRNA after 1 day, and a reduction of 61% in Bcl-2 protein expression [62]. In this study, a cationic lipid carrier was used, and two control oligonucleotides had no effect. Additionally, G3139 had no effect on actin or heat-shock protein 70 (Hsp 70) expression. These cells were then injected subcutaneously into SCID mice. G3139 treatment led to a reduction in tumor weight (59%) and in Bcl-2 protein expression (66–72%). This inhibition was specific as the control reverse and mismatched oligonucleotides were ineffective. Treating the SCID mice with G3139 and dacarbazine, the most effective chemotherapeutic agent for human melanoma, led to a complete and specific ablation of the tumor in three of six animals. The same observations of an increased chemosensitization effect by an oligonucleotide were also demonstrated in N7 and Kato-xIII gastric cancer cells treated either in vitro or when injected subcutaneously into SCID mice [63]. G3139 oligonucleotide at 200 nM, complexed to lipofectin carrier, led to a downregulation of Bcl-2 expression by 50%. This inhibition was believed to be specific: neither two mismatches nor the reverse-sequence oligonucleotide caused a significant decrease in protein expression. However, no Northern blots were presented. These oligonucleotides were also tested in SCID mice containing human gastric cancer tumor xenograft. In SCID mice, a continuous subcutaneously administration at the dose of 10 mg/kg, caused a reduction (approximately 35%) in Bcl-2 expression after 14 days and a moderate diminution in the size of the tumor after 28 days. The control oligonucleotides had no effect. The administration, during the G3139 treatment, of a single intraperitoneal dose of cisplatin (9 mg/kg) on day 7 led to a significant decrease in tumor size (70% compared to treatment with cisplatin or antisense alone), an increase in survival (more than 50%) and an increase (3-fold) in apoptotic cell death. The possible synergism of the G3139/anticancer drug combination has also been demonstrated in other tumors [64–67].

Phase I clinical trials of this combination have also recently been initiated [68,69]. Fourteen patients with advanced malignant melanoma expressing Bcl-2 have been treated with G3139 [68]. The drug was administered by continuous intravenous infusion at doses of between 0.6 and 6.5 mg/kg per day for 14 days. Dacarbazine was administered at a dose of 200 mg/m^2 per day on the fifth and ninth days of G3139 oligonucleotide treatment. The combination treatment was well tolerated, as no-dose limiting toxicities were observed. One patient achieved a complete response, two had partial responses, and three had minor responses. Two patients had stable disease and the six remaining patients showed progressive disease. In ten of the 12 patients evaluable, a clear reduction of Bcl-2 protein expression was demonstrated by Western blots. These in vivo experiments did not require cationic lipids for oligonucleotide delivery, in contrast to the experiments performed in tissue culture. This is usually the case in in vivo assays, and to date it is not well understood why a vector does not appear to be necessary. One hypothesis is that the oligonucleotides interact with circulating proteins, which both protect them against degradation, and serve in ways not yet understood as carriers.

The clinical success so far achieved with G3139 has led to the planning of additional phase I/II trials, in which G3139 efficacy, with or without chemotherapeutic drugs, will be evaluated in various clinical malignancies. Since September 2000, G3139 has also been in pivotal phase III trials for malignant melanoma, chronic lymphocytic leukemia and acute myelocytic leukemia [70]. In addition, a phase III trial was initiated in February 2001 in patients with advanced multiple myeloma in 65 centers in the United States, Canada and Great Britain. The trial will examine whether treatment with G3139 (Genasense) can improve response rates, response duration and quality of life compared with dexamethasone therapy alone.

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