Antisense therapy for malignant mesothelioma with oligonucleotides targeting the *bcl-xl* gene product

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W.R.S. is the recipient of the University of Texas M.D. Anderson Physician-Scientist Award and Grant, and the work was also supported in part by the W.M. Keck Center for Cancer Gene Therapy.

Read at the Eighty-first Annual Meeting of The American Association for Thoracic Surgery, San Diego, Calif, May 6-9, 2001.

Received for publication May 14, 2001; revisions requested July 9, 2001; revisions received Sept 28, 2001; accepted for publication Oct 2, 2001.

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J Thorac Cardiovasc Surg 2002;123:1191-8

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0022-5223/2002 \$35.00+0 12/6/121684

doi:10.1067/mtc.2002.121684

Objective: Malignant pleural mesothelioma is resistant to conventional therapies and to apoptosis. The *bcl-2* family genes are major determinants of apoptotic homeostasis. Malignant pleural mesothelioma lines and tumors rarely express the antiapoptotic Bcl-2 protein but routinely express the antiapoptotic protein Bcl-xl and the proapoptotic proteins Bax and Bak. We have previously shown pharmacologic inhibition of *bcl-xl* expression in malignant pleural mesothelioma can lead to apoptosis, so we sought to determine whether antisense oligonucleotides directed at *bcl-xl* messenger RNA would engender apoptosis, possibly through a “forced imbalance” of *bcl-2* family proteins.

Methods: Malignant pleural mesothelioma lines REN (epithelial) and I-45 (sarcomatous) were exposed to modified *bcl-xl* antisense oligonucleotides directed near the messenger RNA initiation sequence with and without a liposomal delivery system. Untreated cells and *bcl-xl* sense oligonucleotides were controls. Cell viability was measured by colorimetric assay, and apoptosis was evaluated with Hoechst staining and sub-G₁ fluorescence-activated cell sorter analysis.

Results: Bcl-xl protein expression after antisense oligonucleotides was downwardly regulated in both cell lines relative to sense oligonucleotides (>65%). Significant cellular killing in both the I-45 and REN cell lines was achieved with antisense oligonucleotides (compared with sense oligonucleotides) without ($P = .003$ and $.006$, respectively) and with ($P = .006$ and $.0005$, respectively) liposomal delivery. Hoechst staining and sub-G₁ fluorescence-activated cell sorter analysis demonstrated apoptosis to be the mechanism of cellular death. Use of a liposomal delivery system increased therapeutic effect and allowed lower doses of antisense oligonucleotides.

Conclusion: Antisense oligonucleotides directed at the *bcl-xl* gene product engender apoptosis in mesothelioma cell lines. The therapeutic potential of inhibiting expression of this protein in mesothelioma should be evaluated.

Malignant pleural mesothelioma (MPM) continues to be a difficult clinical problem with a high case/fatality ratio. Advances in staging, evaluation, and histopathology have identified a highly selected subgroup that might benefit from aggressive multimodality therapy, but the prognosis for all patients with this disease remains poor.^{1,2} This tumor is clinically resistant to conventional chemotherapy, with no regimen consistently demonstrating a response greater than 20% and most trials demonstrating no significant benefit.³⁻⁵ This is especially discouraging if one considers the fact that metastatic disease (once thought rare) has been noted with increasing frequency as local control measures have improved.^{6,7} Failure of conventional treatment has led

to an interest in novel approaches, such as prodrug gene therapy with the herpes simplex virus thymidine kinase-ganciclovir system and immunotherapy.⁸⁻¹⁰

Another novel approach being explored in cancer treatment, although little studied for MPM, is the forced induction of programmed cell death, or apoptosis. Although the discrete mechanisms of action vary, many conventional treatments depend on an ability to engender apoptosis as a final common pathway. We have previously shown that the specific downward regulation of expression of the *bcl-xl* gene product at both the protein and messenger RNA (mRNA) levels by exposure to a histone deacetylase inhibitor, sodium butyrate, can lead to the development of apoptosis in MPM cells.¹¹ We have also demonstrated that this decrease in *bcl-xl* expression can prime MPM cells for a second proapoptotic stimulus, such as adenoviral transfer of the genes coding for the proapoptotic proteins Bak and Bax.^{12,13} Although seemingly specific for this member of the *bcl-2* family, it is certain that the expression of a number of other genes would be affected by a pharmacologic treatment such as sodium butyrate that inhibits histone deacetylase and allows more accessible complementary DNA for transcription. This raises the question as to whether these previous findings represent a true cause and effect for induction of apoptosis in MPM cell lines or epiphenomena related to other unknown genetic events induced by butyrate exposure. To evaluate this question and elucidate means by which this reproducible finding of Bcl-2 protein family expression can be capitalized on therapeutically, it will be necessary to develop a more specific method to downwardly regulate *bcl-xl* gene expression. In this study we evaluated the ability of an antisense oligonucleotide construct directed near the region of the translation initiation site for the *bcl-xl* messenger RNA to specifically downwardly regulate expression of Bcl-xl protein and lead to apoptotic cellular death in human MPM tumor cell lines.

Methods

Cell Lines and Growth Conditions

To study the effect of *bcl-xl* antisense oligonucleotide exposure in MPM, two well-characterized cell lines were used, I-45 and REN. I-45 is a human sarcomatous type MPM, p53 wild-type (donated generously by Dr Joseph Testa of the Fox Chase Institute, Philadelphia, Pa). REN is a human epithelial type MPM and p53 mutant (developed by author W.R.S.).¹⁴ Cell lines were maintained in Roswell Park Memorial Institute medium containing 10% (volume/volume) fetal bovine serum, 1% nonessential amino acids, and 100- μ g/mL penicillin and 100- μ g/mL streptomycin. Cells were housed in a humidified incubator with 5% carbon dioxide at 37°C.

Antisense Oligonucleotide Constructs and in Vitro Transfection Methods

High-performance liquid chromatography-purified 20-mer *bcl-x* antisense oligonucleotides and complementary-sense oligonucleo-

tides were designed and synthesized (Life Technologies, Inc, Rockville, Md). The antisense oligonucleotide sequence matches the translation-initiation site of human *bcl-x* pre-splice mRNA. The oligonucleotides were designed with a 2'-*o*-(2-methoxy) ethyl modification to assist in prevention of cellular and extracellular enzymatic degradation. The *bcl-x* antisense sequence was as follows: 5'-CCC GGT TGC TCT GAG ACA TT-3'. The *bcl-x* sense sequence was as follows: 5'-AAT GTC TCA GAG CAA CCG GG-3'.

Western Blot Analysis

After MPM cells were exposed to oligonucleotides or control vehicle and cultured for 24, 48, or 72 hours, total cell lysates were prepared by lysing plated cell monolayers with sodium dodecyl-sulfate-polyacrylamide gel electrophoresis sample buffer. The protein content of the lysates was then determined by BCA protein assay (Pierce, Rockford, IL). Then, each lane on a sodium dodecyl-sulfate-polyacrylamide (12%) gel was loaded with 20 μ g of cell lysate and electrophoresed to separate proteins under reducing conditions for the protein of interest. After electrophoresis at 20 mA for 2 hours, the proteins were transferred to high-bond ECL membranes (Amersham Corp, Arlington Heights, Ill). The membranes were then incubated with the primary and secondary antibodies and developed according to the Amersham ECL protocol. Actin was used as a control. Antibodies to actin and Bcl-xl were obtained from Santa Cruz Biotechnology, Inc, Santa Cruz, Calif.

Cellular Viability Assay

Cell viability was tested with the XTT colorimetric assay system (Sigma, St Louis, Mo). After REN and I-45 exposures to oligonucleotides or control at varying doses, the assay evaluated surviving cells at 48 hours after dosing. The labeling reagent was added to the electron-coupling reagent (Roche Diagnostics GmbH, Mannheim, Germany) in a 1:50 ratio. A 50- μ L aliquot of this XTT mixture was then added to each well in a 96-well plate. Plates were then incubated at 37°C for 4 hours. The plates were then analyzed with a colorimetric microplate reader at a wavelength of 450 nm. (DYNEX Technologies, Inc, Chantilly, Va).

Apoptosis Assays

Apoptotic cell death was evaluated by changes in cell morphologic and flow cytometric characteristics. Fluorescence-activated cell sorter analysis was performed as follows: after oligonucleotide exposure and 48-hour culture, cells were trypsin digested, collected by centrifugation, resuspended in Phosphate-buffered saline solution, and fixed in 70% ethanol at 4°C for 1 hour. After centrifugation, the cells were washed in phosphate-buffered saline solution and resuspended in PI staining solution (Roche Diagnostics Corporation, Laboratory Systems, Indianapolis, Ind). Specimens were incubated in the dark for 30 minutes at 37°C and then analyzed with the use of an EPICS Profile II flow cytometer (Coulter Corporation, Hialeah, Fla). An analysis region was set on the basis of the negative controls, and the percentage of sub-G₁ cells was calculated from this region. For Hoechst staining, cells were plated in chamber slides at a concentration of 5000 cells in 500 μ L medium. After 24 hours, the cells were exposed to oligonucleotides. After 72 hours, cells were washed with phosphate-buffered saline solution and then fixed with acetone and acetic acid

Figure 1. Western blot analysis of Bcl-xl protein expression after exposure to *bcl-xl* antisense (ASO) and sense (SO) oligonucleotide constructs. Comparative densitometry demonstrates relative reduction of Bcl-xl protein of 67% in I-45 and 78% in REN cell lines. Actin expression is shown as control and was unaffected by differential treatment.

(3:1). After fixation, Hoechst nuclear staining solution (0.1 $\mu\text{g}/\text{mL}$) was added. Nuclei were then examined with a fluorescent microscope (Nikon Diaphou; Nikon Inc, Melville, NY). Apoptosis was characterized as cells with large segmented nuclei.

Results

Inhibition of Mesothelioma Expression of Bcl-xl Protein by Antisense Oligonucleotide Exposure

Before commenting on the effects of *bcl-xl* antisense oligonucleotide exposure, it is first necessary to demonstrate that the intended effect on the gene product (in this case downward regulation of Bcl-xl protein expression) is noted after exposure. To demonstrate more rigorously whether the *bcl-xl* antisense oligonucleotide is effective in this regard, a comparison was made between Bcl-xl protein expressions following REN and I-45 exposures to antisense oligonucleotide and sense oligonucleotide, rather than to baseline (no oligonucleotide), to assess any effect of nonspecific sense oligonucleotide toxicity on protein production. As is noted in Figure 1, significant decreases in *bcl-xl* expression were noted in REN and I-45 cells after exposure to *bcl-xl* antisense oligonucleotide relative to *bcl-xl* sense oligonucleotide exposure. According to quantitative densitometry, Bcl-xl expression was decreased by 78% in REN (22% of control) and 67% in I-45 (33% of control) cell lines. No effect on actin protein expression was noted.

Mesothelioma Cellular Death After Exposure to *bcl-xl* Antisense Oligonucleotides, Intensified by Liposomal Transfer

A colorimetric (XTT) assay was performed to compare the effects of *bcl-xl* antisense oligonucleotide and sense oligonucleotide exposures on cellular viability in both MPM cell lines. Figure 2, A and B, shows the dose-response effects of exposures to *bcl-xl* antisense oligonucleotide and sense oligonucleotides alone (no liposomes). In the REN and I-45 cell lines, approximately 40% and 50% of cells, respectively, were killed by exposure to antisense oligonucleotide

at the highest dose without liposomes (200 $\mu\text{mol}/\text{L}$), and in both cases the difference of effect between sense and antisense oligonucleotide exposures was significant (200 $\mu\text{mol}/\text{L}$, REN $P = .006$; I-45 $P = .003$, by analysis of variance [ANOVA]). Little overall effect was noted on cellular viability after sense oligonucleotide exposure in either cell line. We then evaluated the effect of *bcl-xl* antisense oligonucleotide and sense oligonucleotide transfer on REN and I-45 cellular viability in a similar fashion as previously, with the use of a commercially available liposomal delivery system (Oligofectamine; Invitrogen Corporation, Carlsbad, Calif), and the results are demonstrated in Figure 3, A and B. There was a significant effect of antisense oligonucleotide treatment and a significant difference noted between *bcl-xl* antisense oligonucleotide and sense oligonucleotide treatments at much lower doses of oligonucleotide, with approximately 60% of REN and 90% of I-45 cells affected (100 nmol/L, REN $P = .0005$, I-45 $P = .006$, by ANOVA). Nonspecific liposomal toxicity effect on cellular viability was noted, with more than 20% of cells killed by the sense oligonucleotide-liposome combination in both REN and I-45 cells.

Apoptosis as the Mechanism for Cellular Death in Mesothelioma Cells Exposed to Antisense Oligonucleotides

To determine whether the cellular death noted previously was due to apoptosis rather than other causes, morphologic and DNA content analyses for apoptosis were then performed. According to Hoechst staining, a significant population of MPM cells exposed to *bcl-xl* antisense oligonucleotide demonstrated fragmentation consistent with apoptosis (data not shown). In an effort to more objectively quantify apoptosis, fluorescence-activated cell sorter cell cycle analysis was performed, evaluating the percentage of cells in sub- G_1 phase after antisense oligonucleotide and sense oligonucleotide exposures with and without liposomal delivery

Figure 2. Cell viability assay (XTT) of mesothelioma cell lines after *bcl-xl* antisense (filled squares) and sense (open squares) oligonucleotide exposures. A, Assay data for REN cell line demonstrating approximately 40% of cells killed by *bcl-xl* antisense oligonucleotide exposure, with little effect noted after *bcl-xl* sense oligonucleotide exposure ($P = .006$ at $200 \mu\text{mol/L}$ by ANOVA). B, Assay data for I-45 cell line demonstrating approximately 50% of cells killed by *bcl-xl* antisense oligonucleotide exposure, with little effect noted after *bcl-xl* sense oligonucleotide exposure ($P = .003$ at $\mu\text{mol/L}$ by ANOVA).

Figure 3. Cell viability assay (XTT) of mesothelioma cell lines after *bcl-xl* antisense (filled squares) and sense (open squares) oligonucleotide exposures with a liposomal delivery system. A, Assay data for REN cell line demonstrating approximately 60% of cells killed by *bcl-xl* antisense oligonucleotide exposure, with approximately 20% of cells killed after *bcl-xl* sense oligonucleotide exposure ($P = .0005$ at 100 nmol/L by ANOVA). B, Assay data for I-45 cell line demonstrating approximately 90% of cells killed by *bcl-xl* antisense oligonucleotide exposure, with approximately 30% of cells killed after *bcl-xl* sense oligonucleotide exposure ($P = .006$ at 100 nmol/L by ANOVA).

systems. Figure 4 demonstrates that apoptosis was present, increased with antisense oligonucleotide relative to sense oligonucleotide, and accentuated with liposomal antisense oligonucleotide transfer.

Discussion

Antisense oligonucleotides have been in use since the late 1970s. Zamencik and colleagues¹⁵ introduced the idea of therapeutic antisense oligonucleotides to prevent neoplastic transformation of cells exposed to the Rous sarcoma virus.¹⁵

These short sequences of nucleotides, generally between 13 and 20 base pairs in length, are constructed to base pair with a specific mRNA sequence of a gene product of interest. By means of one of a number of mechanisms proposed (endonuclease digestion, triplex formation, translational arrest, and others), this base pairing of the oligonucleotide and the mRNA of interest leads to a relatively specific decrease in the expression of the desired protein.¹⁶ Initial enthusiasm regarding this approach was tempered somewhat in the early 1990s by the perception that in vitro results could not

Figure 4. Sub-G₁ fluorescence-activated cell sorter analysis after *bcl-xl* antisense and sense oligonucleotide exposures in I-45 mesothelioma cell line. Level of apoptosis was increased with antisense oligonucleotide treatment and was accentuated when liposomal delivery system (filled bars) was used.

easily be translated in vivo into animal models and human treatments for various diseases because of delivery and toxicity issues.¹⁷ However, more appropriate targets have now been identified, further modifications of backbone structure have been realized, and delivery methods have been improved.¹⁸ In addition to past and ongoing evaluation of antisense oligonucleotides in the laboratory, a number of clinical trials with antisense oligonucleotides have been completed for malignant diseases, with efficacy noted in phase I and phase I-II trials for leukemia (*c-myc* targeting), lymphoma (*bcl-2* targeting), and melanoma (*bcl-2* targeting combined with chemotherapy).¹⁹⁻²¹ More effective targets that may be generated by current efforts in both genomics and proteomics, further refinements in delivery techniques, and alternative constructs such as ribozymes and deoxyribozymes promise to make this strategy more tenable in the future as a means of treating malignant disease.²²

The attraction of the *bcl-2* family of genes and gene products for treatment lies in the fact that these proteins help to control one of the final common pathways for a variety of apoptotic stimuli, namely the release of cytochrome c from the mitochondrial membrane and activation of caspases.²³ The Bcl-2 family is a group of more than 15 proteins that, through relationships with one another as well as other constituents of programmed cell death pathways, regulate apoptotic homeostasis in cells. The longer splice product of the *bcl-x* gene (located on the short arm of chromosome 20; 20pter-p12.1), Bcl-xl, is one of the more important anti-apoptotic members of this family. Like Bcl-2, this protein seems to function on a basic level as an ion-type channel

regulator of mitochondrial membrane stability.²⁴ The Bcl-xl protein has been shown to bind directly to the porin voltage-dependent ion channel protein and to prevent cytochrome c release from the mitochondrion, even after proapoptotic stimuli.²⁵ Unlike Bcl-2, it does not seem to require dimerization with proapoptotic proteins such as Bax to provide apoptosis prevention, although it can interact with a number of these proteins, including Bax, Bak, and Bad.²⁶ It has been demonstrated that Bcl-xl is at least as potent as Bcl-2 in the prevention of apoptosis in a wide variety of human malignant cell lines when challenged with cytotoxic and proapoptotic stimuli.²⁷ Interestingly, Bcl-xl appears to be much more important developmentally in mammals, because *bcl-x*-deficient mice undergo massive central neural and hematopoietic apoptosis, with resultant embryonic death, whereas *bcl-2*-deficient strains survive.²⁸

Several investigators have noted a consistent alteration of Bcl-2 family expression pattern in MPM. Narasimhan and associates²⁹ noted that Bcl-2 expression was absent in 13 of 17 MPM cell lines and present only during mitosis in the 3 of 17 that did have positive results. In this same study, Bax expression was uniform in all lines. Similarly, Segers and colleagues³⁰ noted a lack of expression of Bcl-2 in 57 of 62 MPM tumor specimens, and Soini and coworkers³¹ demonstrated that 7 of 7 MPM cell lines and 28 of 35 tumor specimens lacked expression of Bcl-2, although in all lines and tumors Bcl-xl expression was intact. The findings that expression of only one of these antiapoptotic proteins is common (Bcl-2 rare, Bcl-xl common) and that expressions of all major proapoptotic proteins, such as Bax and Bak, are

intact may indicate a potential therapeutic target—downward regulation of expression of *bcl-xl* with resultant “unopposed” proapoptotic protein expression.

Our previous work has suggested a critical role for *bcl-xl* gene expression in MPM cell lines with respect to apoptotic maintenance. We have demonstrated that pharmacologic downward regulation of *bcl-xl* expression at both the protein and mRNA levels by a histone deacetylase inhibitor, sodium butyrate, can lead to apoptotic cellular death. Supporting these results is an experiment demonstrating that when we caused overexpression of *bcl-xl* in MPM cells by stable plasmid transfer that the sensitivity to butyrate was abrogated, and this insensitivity was proportional to level of Bcl-xl protein production.¹¹ We theorize that the “forced imbalance” of *bcl-2* family proteins by Bcl-xl downward regulation in this situation leads to relatively unopposed proapoptotic protein expression and therefore ultimately to apoptosis. In further support of this hypothesis, we have shown that upward regulation of proapoptotic Bak protein expression in these MPM cell lines by use of a binary adenoviral gene therapy vector system may engender apoptosis, with other Bcl-2 family member levels of protein expression unaffected.³² In earlier experiments we demonstrated an apparently specific effect of sodium butyrate on a single member of the Bcl-2 family in mesothelioma.¹¹ The obvious concern, however, in any experiment involving the exposure of cells to pharmacologic agents is the effect on genes or proteins that are not being evaluated. This concern is compounded by the fact that sodium butyrate is a histone deacetylase inhibitor. These agents actually inhibit the re-winding of DNA onto histones when it is not actively being transcribed and are therefore usually associated with upward regulation of gene transcription rather than downward regulation. To address these concerns, in this series of experiments we sought to regulate *bcl-xl* gene expression downward more specifically through antisense techniques targeting the *bcl-xl* mRNA.

Other investigators have demonstrated the therapeutic ability of antisense oligonucleotides directed at the *bcl-xl* gene product. Leech and coworkers,³³ demonstrated the ability of modified *bcl-xl* antisense oligonucleotides to induce apoptosis in lung cancer cell lines. However, this was effective only in lines lacking *bcl-2* expression and was ineffective in lines expressing both Bcl-xl and Bcl-2 (cogent to the situation with MPM cells and tumors). This group later demonstrated that a bispecific *bcl-2/bcl-xl* antisense oligonucleotide was necessary to engender apoptosis in tumor cell lines expressing both proteins.³⁴ Overexpression of *bcl-xl* has been noted as well in breast carcinoma and in gastric carcinoma (with absence of Bcl-2 protein expression), and again apoptotic cellular death has been engendered by exposure to a *bcl-xl* antisense oligonucleotide.^{35,36}

The finding that the *bcl-xl* antisense oligonucleotide con-

struct used here was more effective at a lower dose when combined with a liposomal transfer system is not surprising. Liposomal delivery systems have been known for quite some time to enhance cellular uptake of oligonucleotides in vitro, especially those that are cationic.^{18,37} Although antisense oligonucleotides have been known for quite some time to be effective inhibitors of specific mRNA products, an adenoviral vector capable of transferring the antisense construct could be considered as an alternative and would give additional assurance of cellular entry. An adenoviral vector paradigm could have certain other advantages as well, including avoidance of lysosomal degradation and greatly increased copy number of the antisense construct. Various constructs have been used both in vitro and in vivo to treat non-small-cell lung cancer (anti-K-ras), gastric carcinoma (anti-endothelial growth factor receptor), glioma (anti-urokinase type plasminogen activator), and small cell lung carcinoma (anti-c-kit).³⁸⁻⁴¹

What about the clinical application of *bcl-xl* antisense oligonucleotides to the treatment of this disease? As has been noted with a variety of gene therapy techniques, combination therapies with conventional modalities plus antisense may be necessary. We have noted previously that pharmacologic inhibition of *bcl-xl* could prime MPM cells for a second proapoptotic stimulus (proapoptotic gene transfer of the *bak* or *bax* gene).^{12,13} Other investigators have noted a relationship between Bcl-xl expression and chemotherapy sensitivity. In one study, U-937 cells were stably transfected to overexpress Bcl-xl, and apoptosis was decreased after exposure to vinblastine, paclitaxel, and cisplatin by 67%, 51%, and 55%, respectively.⁴² In a system designed to provide lymphoma cells with microenvironment survival adjuvants (CD40 antibody, interleukin 4, vascular cell adhesion molecule 1 fusion protein) followed by etoposide, the most consistent change that correlated with chemotherapy resistance was upward regulation of Bcl-xl expression. Expected downward regulation of proapoptotic proteins Bak and Bax were not noted.⁴³ Similarly, Luo and associates⁴⁴ demonstrated that hepatoblastoma cells were rendered more sensitive to paclitaxel and doxorubicin by stable transfection of a *bcl-xl* antisense construct. When colonic adenocarcinoma cells noted be deficient in Bcl-2 but to overexpress Bcl-xl were treated with *bcl-xl* antisense oligonucleotides followed by 5-fluorouracil, they exhibited a 40% decrease in cell viability and a 55% increase in apoptosis relative to 5-fluorouracil alone.⁴⁵ Finally, stable *bcl-xl* overexpression in a murine tumor cell line (FK5.12) has been shown to engender a form of multidrug resistance phenotype, with these cells dramatically more resistant than parental cells to a panel of six different chemotherapeutic agents (bleomycin, vincristine, cisplatin, etoposide) and metabolic poisons (hygromycin B, mycophenolate).⁴⁶ We are currently investigating the ability of this and other *bcl-xl*

antisense constructs to act in an additive or synergistic fashion with conventional chemotherapeutic agents in MPM cells and with the transfer of proapoptotic genes. According to our studies to date, *bcl-xl* expression appears to be a viable target for therapeutic investigation in MPM, and continued investigation is warranted.

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Discussion

Dr David S. Schrump (Bethesda, Md). I have several questions relating to duration of exposure, resistance, and so forth. I looked at the growth inhibition assays, and there is a nice response.

It seems to be dose related. Have you looked at the minimum duration that is required to achieve apoptosis? As a follow-up, have you looked to see what happens to the cells that become resistant to the antisense effect? Are you able to achieve 100% killing if you modify the conditions? If not, what is the phenotype of the cells that are resistant?

Dr Smythe. In answer to the first question, the cellular viability effect and apoptosis both appear to peak somewhere between 24 and 48 hours. Beyond that time, exposure really does not do much more. Actually, the cells will begin to regrow. With respect to the phenotype or the genotype of the cells that are resistant to antisense, we have not examined that.

Dr Schrump. Are the doses that you used comparable with what has been achieved in the phase I studies?

Dr Smythe. Yes, they are comparable, especially with the liposomal delivery system. Interestingly enough, of the two phase I studies that have been reported, neither used a liposomal delivery system. They just used oligonucleotide alone. But these doses are definitely comparable.

Targeted

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