Activity of a Novel bcl-2/bcl-xL-Bispecific Antisense Oligonucleotide Against Tumors of Diverse Histologic Origins

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Background: **Increased expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL is involved in the development and progression of many tumors. We recently reported that the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 induces apoptosis in lung carcinoma cells. To further assess the therapeutic potential of oligonucleotide 4625, we investigated its effect on a series of human tumor cell lines of diverse histologic origins *in vitro* and *in vivo*. *Methods:* Oligonucleotide 4625-mediated inhibition of bcl-2 and bcl-xL expression *in vitro* was measured in breast carcinoma cells with the use of reverse transcription–polymerase chain reaction (PCR), real-time PCR, and western blotting. Cytotoxicity was assessed in several different cell lines by measurement of tumor cell growth, propidium iodide uptake, and nuclear apoptosis. The *in vivo* activity of oligonucleotide 4625 was determined by the inhibition of growth of established tumor xenografts in nude mice, immunohistochemical staining of Bcl-2 and Bcl-x proteins in the tumors, and western blotting of tumor lysates. Apoptosis in tumor xenografts was detected with the use of *in situ* TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining. All statistical tests are two-sided. *Results:* In breast carcinoma cells, oligonucleotide 4625 treatment reduced bcl-2 and bcl-xL messenger RNA levels in a dose-dependent manner. At 600 n*M*, oligonucleotide 4625 reduced Bcl-2 and Bcl-xL protein levels to 25% (95% confidence interval [CI] = 16% to 34%) and 20% (95% CI = 14% to 26%), respectively, of the levels in untreated cells and it decreased viability in all cell lines mainly by inducing apoptosis. *In vivo*, oligonucleotide 4625 statistically significantly inhibited the growth of breast and colorectal carcinoma xenografts by 51% (95% CI = 28% to 74%) and 59% (95% CI = 44% to 74%), respectively, relative to those treated with control oligonucleotide 4626; it also reduced Bcl-2 and Bcl-xL protein levels and induced tumor cell apoptosis. *Conclusion:* The bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 merits further study as a novel compound for cancer therapy. [J Natl Cancer Inst 2001;93: 463–71]**

Impaired apoptosis due to blockade of the cell death-signaling pathways is involved in tumor initiation and progression, since apoptosis normally eliminates cells with damaged DNA or aberrant cell cycling, i.e., cells with increased malignant potential. From this recognition, a concept emerged that elevation of the threshold at which apoptosis is initiated represents a central step for tumor development and drug resistance. The structurally related Bcl-2 family proteins either inhibit or promote apoptosis, thereby regulating cell death in a rheostatic manner *(1).* The anti-apoptotic family members Bcl-2 and Bcl-xL both localize to cellular membranes, particularly in mitochondria, where they stabilize the transmembrane potential and reduce membrane permeability. This action inhibits the release from mitochondria of substances involved in either signaling or execution of apoptosis, such as cytochrome c, procaspase 3, and apoptosis-inducing factor *(2).* Although the issue is currently disputed, Bcl-xL may also inhibit apoptosis by binding to Apaf-1, thereby preventing its association with procaspase 9 and cytochrome c and the subsequent activation of downstream effector caspases *(3).* Although Bcl-2 and Bcl-xL seem to be functionally indistinguishable, there is evidence that they differ in their ability to protect cells from different apoptotic stimuli *(4,5).* In addition, Bcl-2 seems to contribute to tumor cell survival by diminishing the rate of cell proliferation *(6)* and by allowing tumor cells to escape from destruction by effector cells of the immune system *(7).*

The majority of solid tumors are protected by at least one of the two cell death antagonists, Bcl-2 or Bcl-xL. Although the relative expression levels of Bcl-2 and Bcl-xL vary, depending on the type and stage of the tumor, evidence for Bcl-2 and Bcl-xL overexpression as negative prognostic markers is provided by several findings: 1) Bcl-2 expression in non-small-cell lung carcinoma and estrogen receptor-positive breast carcinoma is associated with lymph node invasion *(8,9);* 2) Bcl-xL expression in breast and colorectal carcinomas is associated with advanced-stage disease *(10,11);* 3) Bcl-2 and Bcl-xL expression in prostate carcinoma is associated with tumor grade, metastatic potential, recurrence after prostatectomy, and radioresistance *(12,13);* and 4) overexpression of Bcl-2 in cutaneous malignant melanoma is associated with shorter survival *(14).* In tumors where Bcl-2 and Bcl-xL are coexpressed, it is difficult to predict which of the two proteins is more critical for survival and, thus, the more relevant target for gene therapy approaches. Moreover, tumor cells are able to switch expression from Bcl-2 to Bcl-xL *(15),* and forced overexpression of Bcl-2 has been reported to result in the reciprocal inhibition of Bcl-xL expression *(16).* In

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view of this complexity, the simultaneous inhibition of Bcl-2 and Bcl-xL expression in tumors by a single compound represents an appealing concept.

Antisense therapy holds the promise for selective silencing of genes, e.g., those involved in the resistance of cells to apoptosis induction, such as bcl-2 or bcl-x. Antisense oligonucleotides act by specifically hybridizing with complementary messenger RNA (mRNA) sequences based on Watson–Crick base pairing, thereby inhibiting gene expression primarily by activation of ribonuclease (RNase) H, which subsequently cleaves the target mRNA *(17).* Second-generation antisense oligonucleotides with a phosphorothioate backbone and up to five nucleotides at both the 5′ and the 3′ end modified by methoxy-ethoxy (MOE) residues at the 2′-α-position of the deoxyribose show enhanced RNA-binding affinity compared with their first-generation 2′-deoxy counterparts. Moreover, owing to the central 2′-deoxy gap, these antisense compounds are still capable of activating RNase H *(18).* Antisense oligonucleotides complementary to either the bcl-2 or the bcl-xL mRNA have previously demonstrated activity against tumor cells of various origins *(19–24).* Bcl-2 antisense oligonucleotides in combination with chemotherapy are currently in phase I/II clinical trials for the treatment of patients with lymphoma and malignant melanoma, and further trials with patients with lung, prostate, renal, or breast carcinoma are ongoing or planned.

Based on the assumption that simultaneous inhibition of bcl-2 and bcl-xL expression with a single compound might be therapeutically more promising than the inhibition of either alone, we have recently designed the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 and reported its ability to inhibit the expression of bcl-2 and bcl-xL and to induce apoptosis in lung carcinoma cells *in vitro (25).* In the present study, we investigated the ability of antisense oligonucleotide 4625 to inhibit the expression of bcl-2 and bcl-xL in a representative breast carcinoma cell line and to inhibit the growth and induce death in cell lines from lung, breast, colorectal, and prostate carcinomas and from malignant melanoma *in vitro.* In addition, the ability of oligonucleotide 4625 to inhibit tumor growth and to reduce the Bcl-2 and Bcl-xL proteins *in vivo* was examined in established tumor xenografts in nude mice. Our data demonstrate the potent antitumor activity of oligonucleotide 4625 against a series of tumor cell lines *in vitro* and *in vivo* and suggest its use in cancer therapy.

Materials and Methods

Cell Lines

The following nine human tumor cell lines of different histologic origins were obtained from commercial cell culture collections unless indicated otherwise: SW2 (small-cell lung carcinoma), NCI-H125 (non-small-cell lung carcinoma), MDA-MB-231 (estrogen-independent breast adenocarcinoma), MCF-7 (estrogen-dependent breast adenocarcinoma), Colo-320 (colorectal adenocarcinoma), LoVo (colorectal adenocarcinoma), PC-3 (prostate adenocarcinoma), C4–2 (prostate adenocarcinoma; provided by Dr. G. Thalmann, University Hospital, Bern, Switzerland), and A-375 (cutaneous malignant melanoma; provided by Dr. R. Dummer, University Hospital, Zurich, Switzerland). MDA-MB-231 cells were cultured in high-glucose Dulbecco′s modified Eagle medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin. All other cell lines were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, 2 m*M* L-glutamine, 2.5 g/L glucose, 10 m*M* HEPES, 1 m*M* sodium pyruvate, 50 IU/mL penicillin, and 50 μg/mL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. All cell culture reagents were obtained from Hyclone Europe, Ltd. (Cramlington, U.K.).

Oligonucleotides

Antisense oligonucleotide 4625 binds to a region of high homology shared by bcl-2 and bcl-xL comprising nucleotides 605–624 and 687–706 of the two mRNAs, respectively *(25).* These target sites are located 573 and 550 nucleotides downstream of the AUG start site of the bcl-2 and bcl-xL mRNA, respectively. Oligonucleotides 4626 and 4258 were used as scrambled sequence controls. All oligonucleotides were 20-mers with a phosphorothioate backbone and nucleotides at the 5′ and 3′ ends modified by MOE residues at the 2′-α-position of the deoxyribose (represented by underlined letters in the sequences shown below). Oligonucleotides were synthesized as described previously *(26)* on an Oligopilot II (Amersham Pharmacia Biotech, Uppsala, Sweden) at a 150- to 180-μmol scale on a polystyrene primer (Amersham Pharmacia Biotech) derivatized via a succinyl arm by the first corresponding 2′-MOE 3′ nucleoside. Crude oligonucleotides were purified by reverse-phase high-performance liquid chromatography. Purity was assessed by capillary gel electrophoresis, the phosphodiester content was checked with ^{31}P-nuclear magnetic resonance, and the oligonucleotides were characterized by MALDI-TOF MS (i.e., matrix-assisted laser desorption ionization time of flight mass spectrometry). Oligonucleotides displayed a length purity higher than 95% with a phosphodiester content of less than 0.3%. The sequences were as follows: 4625, 5′-AAGGCATCCCAGCCTCCGTT-3′ (antisense); 4626, 5′-CACGTCACGCGCGCACTATT-3′ (control); and 4258, 5′-CATATCACGCGCGCACTATG-3′ (control).

Treatment of Cells With Oligonucleotides

In vitro, oligonucleotides were delivered to the cells in the form of complexes with the transfection reagent Lipofectin (Life Technologies, Inc. [GIBCO BRL], Glasgow, U.K.) as described previously *(25).* Lipofectin (50 μg/mL) was allowed to complex with oligonucleotides, and this solution was further diluted to the desired concentration in serum and antibiotic-free medium before addition to the cells. Except for the growth assays described below, cells were incubated with oligonucleotides and Lipofectin for 20 hours, after which the Lipofectin solution was replaced by standard cell culture medium as described above.

Reverse Transcription–Polymerase Chain Reaction

One microgram of total RNA was reversed transcribed for 1 hour at 37 °C in 20-μL reactions containing 50 m*M* Tris (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 500 μ*M* each deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTTP), 7.5 μg/mL random hexamers (Promega Corp., Madison, WI), 10 m*M* dithiothreitol, and 200 U Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.). For the detection of bcl-2 mRNA, 2 μL of the reverse-transcribed material was amplified by polymerase chain reaction (PCR) in 50-μL reactions containing 10 m*M* Tris (pH 8.3), 50 m*M* KCl, 2.5 m*M* $MgCl_2$, 200 μ*M* each dATP, dGTP, dCTP, and dTTP, 0.2 μ*M* of each primer, and 0.25 U Amplitaq DNA polymerase (Applied Biosystems [formerly Perkin-Elmer Applied Biosystems], Foster City, CA), for a total of 30 cycles consisting of 94 °C for 45 seconds, 55 °C for 60 seconds, and 72 °C for 60 seconds, with a final extension step for 10 minutes at 72 °C. Detection of bcl-xL mRNA was done under similar conditions, except that 1.5 m*M* $MgCl_2$ was used in the PCR reaction, and cycles were reduced to 28. To control for the presence and quantity of RNA in each reaction, we reamplified 5 μL of each PCR for 33 cycles under similar conditions with primers for β2-microglobulin able to discriminate between complementary DNA (cDNA) and genomic DNA. Amplification products were analyzed by electrophoresis in ethidium bromide-stained agarose gels. Primer sequences were as follows: bcl-2, 5′-GTGAACTGGGGGAGGATTGT-3′ (forward) and 5′-GGAGAAATCAAACAGAGGCC-3′ (reverse); bcl-xL, 5′-CCCAGAAAGGATACAGCTGG-3′ (forward) and 5′-GCGATCCGACTCAC-CAATAC-3′ (reverse); and β2-microglobulin, 5′-GTGGAGCAT TCAGACTT-GTCTTTCAGC-3′ (forward) and 5′-TTCACTCAATCCAAATGCGGCATCTTC-3′ (reverse).

Real-Time PCR

Total RNA was isolated from cells by use of the RNeasy Mini Kit (Qiagen AG, Basel, Switzerland). For cDNA synthesis, Taqman reverse transcription reagents were used as described in the user's manual of the ABI Prism 7700 Sequence Detection system, which was used for real-time PCR amplification following the Taqman Universal PCR Master Mix protocol (Perkin-Elmer Applied Biosystems). Relative quantification of gene expression was performed as

described in the manual with the use of ribosomal RNA (rRNA) as an internal standard and the comparative threshold cycle method. Briefly, 0.5 μg of total RNA was used to generate cDNA by the reverse transcription reaction. The bcl-2 and bcl-xL cDNAs were amplified with the use of primers and Taqman probes that had been selected with the use of the Primer Express Applications-Based Primer Design Software (Perkin-Elmer Applied Biosystems). For bcl-2, primers with the sequences 5′-CATGTGTGTGGAGAGCGTCAA-3′ and 5′-GCCGGTTCAGGTACTCAGT CA-3′ were used, together with a Taqman probe of sequence 5′-CCTGGTGGACAACATCGCCCTGT-3′. For bcl-xL, primers with the sequences 5′-TCCTTGTCTACGCTTTCCACG-3′ and 5′-GGTCGCAT TGTGGCCTTT-3′ were used together with a Taqman probe of sequence 5′-ACAGTGCCCCGCCGAAGGAGA-3′. The bcl-2 and bcl-xL probes were labeled at the 5′ end with the reporter dye 6-carboxy-fluorescein and at the 3′ end with the quencher 6-carboxy-tetramethyl-rhodamine. A Basic Local Alignment Search Tool (BLAST) search of the National Center for Biotechnology Information (NCBI) database containing all sequences in the GenBank, European Molecular Biology Laboratory (EMBL) database, and the DNA Database of Japan (DDBJ) revealed no homology of the primer and probe sequences to any other known human genes. Data are presented relative to an untreated control sample chosen as calibrator. The range of a given sample relative to the calibrator was always less than 10 %.

Western Blot Analysis

Lysates from cells treated with oligonucleotides were subjected to western blot analysis as described previously *(23).* Twenty micrograms of soluble protein per sample was separated by 12% polyacrylamide gel containing sodium dodecyl sulfate and transferred to a polyvinylidene fluoride membrane in a semidry blotting chamber for 1 hour. The blots were blocked in Tris-buffered saline containing 5% bovine serum albumin (BSA) and 5% nonfat dry milk and then incubated overnight at 4 °C with mouse anti-human Bcl-2 monoclonal antibody (Dako Diagnostics AG, Glostrup, Denmark) or rabbit anti-human Bcl-x polyclonal antibody (Transduction Laboratories, Lexington, KY). Actin, stained with a mouse anti-actin monoclonal antibody (ICN Biomedicals, Inc., Aurora, OH), was used as a loading control. For the detection of the primary antibodies, we incubated blots with rabbit anti-mouse or goat anti-rabbit immunoglobulin (Ig) peroxidase conjugates (Sigma Chemical Co., St. Louis, MO), respectively, for 1 hour at room temperature. Antibodies were added in Tris-buffered saline containing 1% BSA, 1% nonfat dry milk, and 0.05% Tween 20 (Sigma Chemical Co.). Visualization of the immunocomplexes was performed by enhanced chemiluminescence with the use of the ECL™ Kit (Amersham Pharmacia Biotech), followed by exposure to x-ray films. Relative protein levels were quantified with the use of Scion software (Scion Corp., Frederick, MD) on scanned films.

Measurement of Cell Growth Inhibition

Effects of oligonucleotides on cell growth were determined as described previously *(23)* with the use of a colorimetric assay based on the reduction of the tetrazolium salt MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by mitochondrial dehydrogenases in viable cells. For each experiment, cells were seeded in 96-well plates and cultured for 24 hours. Oligonucleotides were then added in the form of complexes with Lipofectin up to a total volume of 100 μL, and cells were incubated at 37 °C. Seventy-two hours after the treatment was started, 10 μL of MTT reagent (10 mg/mL) was added and allowed to react for 90 minutes at 37 °C before the addition of 100 μL of solubilization reagent (20% sodium dodecyl sulfate in 50% dimethyl formamide [pH 4.7] and pH-adjusting solution [i.e., 80% acetate and 20% 1 *M* HCl]). Substrate cleavage was monitored 12 hours later at 570 nm by use of a SPECTRAmax 340 microplate reader and analyzed by use of SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Measurement of Cell Death and Apoptosis

Tumor cell death and apoptosis were measured 72 hours after the start of treatment with 600 n*M* oligonucleotide by flow cytometry with the use of a FACScalibur flow cytometer and the CellQuest software (Becton Dickinson, Mountain View, CA). Cells were incubated with 10 μg/mL propidium iodide for 5 minutes, and the fraction of cells with increased fluorescence intensity was measured. Cell debris was excluded from the analysis by appropriate light scatter gating. The apoptotic fraction was determined after the cells were made permeable with 50% ethanol for 30 minutes followed by incubation with 5 μg/mL RNase A for 30 minutes and incubation with 40 μg/mL propidium iodide for 10 minutes. After clusters and fragments were excluded *(27),* cells with sub-G_0/G_1 DNA profiles were identified. In addition, apoptosis was assessed by nuclear staining with Hoechst 33342 dye (Sigma Chemical Co.). For this purpose, cells were pelleted, resuspended, and fixed in 4% paraformaldehyde–0.05% saponin–5 μg/mL Hoechst 33342 for 15 minutes before cytospin centrifugation (20*g* for 3 minutes at room temperature). Cells were mounted with Mowiol (Calbiochem, La Jolla, CA), and nuclei were observed by use of a Leica confocal laserscan microscope equipped with SCANware software (Leitz, Wetzlar, Germany). Images were processed with the use of the Imaris software (Bitplane, Zurich, Switzerland).

Determination of Antitumor Activity *In Vivo*

Six- to 8-week-old female CD1 nu/nu (nude) mice were obtained from BRL (Fullinsdorf, Switzerland) and kept under specific-pathogen-free conditions. Animal experiments were performed according to the guidelines of the Veterinary Office of Zurich, Switzerland. Tumor xenografts were raised by subcutaneous injection of 10^7 MDA-MB-231 or Colo-320 cells into the lateral flanks of the mice under ether anesthesia. Growing tumors were measured with calipers, and the approximate tumor volume was calculated by use of the following formula: volume (mm^3) = length × width^2/2. Once tumors had reached an average size of 75 mm^3, mice were randomly assigned to treatment groups matched for equal mean tumor volumes. Oligonucleotides were injected intraperitoneally at a dose of 20 mg/kg per day over a period of 3 weeks. MDA-MB-231 tumors were measured after treatment for 0, 8, 13, 18, and 22 days, while Colo-320 tumors were measured after 0, 6, 11, 15, and 21 days.

Detection of Bcl-2 and Bcl-xL Proteins in Tumor Xenografts

After 2 weeks of treatment as described above, mice were killed by cervical dislocation, and their tumors were removed, immersed in Hanks' balanced salt solution, and snap-frozen in liquid nitrogen. One half of each tumor was analyzed by immunohistochemical staining for Bcl-2 and Bcl-x; the other half was lysed and used for western blotting of Bcl-2 and Bcl-xL.

For immunohistochemistry, 5-μm tissue sections were cut in a cryostat, placed on siliconized glass slides, air-dried, fixed with acetone for 10 minutes, and stored at −70 °C. Bcl-2 was stained by incubating rehydrated sections with primary fluoresceinated mouse monoclonal antibodies against human Bcl-2 (clone 124; Dako Diagnostics AG), followed sequentially by rabbit polyclonal anti-fluorescein isothiocyanate antibodies (Dako Diagnostics AG) and alkaline phosphatase-labeled donkey anti-rabbit Ig antibodies (Jackson Laboratories, West Grove, CA). Bcl-x was stained with the use of polyclonal rabbit anti-Bcl-x antibodies (Transduction Laboratories). Primary rabbit antibodies were revealed by sequential incubation with alkaline phosphatase labeled goat anti-rabbit Ig antibodies and donkey anti-goat Ig antibodies (Jackson Laboratories). Dilutions of the affinity-purified secondary and tertiary antibodies were made in Tris-buffered saline containing 5% normal mouse serum. Alkaline phosphatase was visualized with the use of naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. Color reactions were performed at room temperature for 15 minutes with reagents (Sigma Chemical Co.). Sections were counterstained with hemalum, and coverslips were mounted with glycerol and gelatin.

For the determination of the levels of Bcl-2 and Bcl-xL proteins in tumor lysates, tumors were homogenized with the use of a Polytron PT 3000 (Kinematica AG, Littau, Switzerland), and protein extraction and immunoblotting were performed as described above under the heading entitled "Western Blot Analysis."

Detection of Apoptosis in Tumor Xenografts

For TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining, formalin-fixed and paraffin-embedded tumor sections (4 μm) were deparaffinized and rehydrated by standard procedures. The tissues were rinsed briefly in Tris-buffered saline (i.e., 0.15 *M* NaCl and 50 m*M* Tris–HCl [pH 7.4]) and digested with 30 μg/mL proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) in Tris-buffered saline for 20 minutes at room temperature. After being washed in distilled water for 10 minutes, the sections were equilibrated for 15 minutes in TdT reaction buffer (i.e., 200 m*M* potassium cacodylate, 2.5 m*M* $CoCl_2$, 0.25

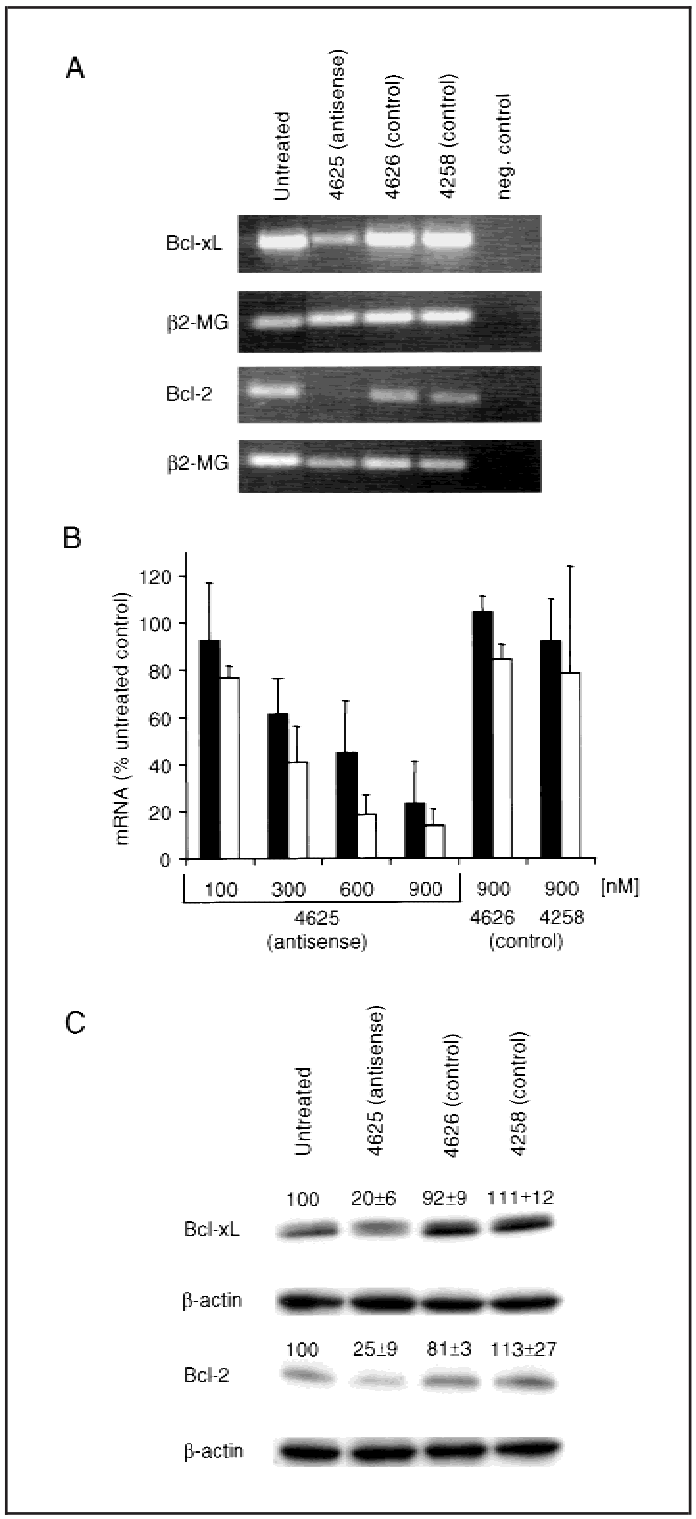

Fig. 1. Inhibition of bcl-2 and bcl-xL messenger RNA (mRNA) and protein expression in MDA-MB-231 breast carcinoma cells. **A**) Cells were treated for 20 hours with antisense oligonucleotide 4625 or control oligonucleotides 4626 and 4258 at a concentration of 600 n*M*. Cells were harvested, and mRNA levels of bcl-xL and bcl-2 were determined by reverse transcription–polymerase chain reaction. β2-Microglobulin (β2-MG) was used as a control for RNA quantity and integrity. Bands are shown from one representative experiment of three experiments. **B**) Cells were treated for 20 hours with antisense oligonucleotide 4625 or control oligonucleotides 4626 and 4258 at the concentrations indicated (n*M*). Cells were harvested, and mRNA levels of bcl-xL (**solid bars**) and bcl-2 (**open bars**) were assessed by reverse transcription followed by real-time PCR quantification. The levels in untreated cells measured under identical experimental conditions were taken as 100%. Data represent the mean and 95% confidence interval (n = 3). **C**) Cells were treated for 40 hours with 600 n*M* antisense oligonucleotide 4625 or control oligonucleotides 4626 or 4258. Cells were harvested, and Bcl-2 and Bcl-xL protein levels were determined by western blotting. Bands from one representative experiment are shown; **numbers** indicate the mean and 95% confidence interval (n = 3) given as percentage of the Bcl-2 and Bcl-xL levels in untreated cells. β-Actin was used as a loading control. Bcl-xL migrated at 31 kilodaltons (kd), Bcl-2 at 28 kd, and β-actin at 48 kd.

mg/mL BSA, and 25 mM Tris–HCl [pH 6.6]). The sections were then covered with the TUNEL reagent mix (62.5 U/mL calf thymus TdT [Boehringer Mannheim GmbH] and 3.75 nmol/mL digoxigenin-11-deoxyuridine triphosphate in TdT reaction buffer) and incubated for 1 hour at 37 °C in a water-saturated atmosphere. The sections were washed for 10 minutes with 2× standard saline citrate at 40 °C, rinsed with Tris-buffered saline, and preincubated with blocking buffer (2% BSA and 0.3% Triton X-100 in Tris-buffered saline) for 30 minutes at room temperature. For the detection of digoxigenin, sections were treated for 1 hour with alkaline phosphatase-labeled Fab fragments of affinity-purified sheep anti-digoxigenin antibodies (1 U/mL blocking buffer at room temperature). After a further washing step with Tris-buffered saline, the red reaction product was developed under visual control with the use of naphthol AS-BI phosphate and new fuchsin as substrate. Endogenous alkaline phosphatase was blocked by levamisole. After being counterstained with hemalum, coverslips were mounted with glycerol–gelatin. Tissue sections from human tonsils, typically showing apoptotic cells, were used as positive controls.

Statistical Analysis

If not stated otherwise, data represent the mean and 95% confidence interval (CI) of at least three independent determinations. *In vitro* growth assays were analyzed with the use of analysis of variance (ANOVA) for repeated measurements with the two within-factors concentration and treatment. Paired *t* tests were used to determine the statistical significance of the antisense effect on cell death compared with the control treatment, *P* values of less than .05 were considered to be statistically significant, and all statistical tests were two-sided. The effect of antisense oligonucleotide 4625 and control oligonucleotide 4626 on tumor growth in mice was analyzed with the use of ANOVA for repeated measurements. Within-factor time and between-factor treatment were compared by use of Bonferroni–Dunn *post-hoc* tests.

RESULTS

Inhibition of bcl-2 and bcl-xL mRNA and Protein Expression by Oligonucleotide 4625

Oligonucleotide 4625 is 100% complementary to the bcl-2 mRNA and has three base mismatches to the bcl-xL mRNA, two of which were modified by 2′-MOE residues. Based on this design, it has the potential to hybridize to both mRNAs and to inhibit the expression of both genes. To demonstrate this bispecific activity, we treated MDA-MB-231 breast carcinoma cells expressing high levels of Bcl-2 and Bcl-xL with oligonucleotides, and we determined bcl-2 and bcl-xL mRNA by reverse transcription–PCR. Samples treated with antisense oligonucleotide 4625 showed markedly reduced levels of both bcl-2 and bcl-xL mRNAs compared with untreated samples or samples treated with control oligonucleotide (Fig. 1, A). For precise quantification of sequence-specific inhibition of mRNA expression, real-time PCR was performed. Antisense oligonucleotide 4625 reduced bcl-2 and bcl-xL mRNA in a dose-dependent manner, as determined 20 hours after the start of transfection (Fig. 1, B). The maximal inhibition was achieved with 900 n*M* oligonucleotide 4625 and was 86% (95% CI = 79% to 93%) and 77% (95% CI = 60% to 94%) for bcl-2 and bcl-xL, respectively. The concentrations at which mRNA expression was inhibited by 50% (IC_{50} values) were 250 n*M* and 500 n*M* (data not

shown). The control oligonucleotides 4626 and 4258 had only marginal effects on bcl-2 and bcl-xL mRNA expression, indicating that the bispecific activity of oligonucleotide 4625 was sequence specific and was not related to unspecific gene-regulatory events. On the protein level, 600 n*M* oligonucleotide 4625 reduced Bcl-2 and Bcl-xL expression to 25% (95% CI = 16% to 34%) and 20% (95% CI = 14% to 26%), respectively, when measured 40 hours after the start of transfection (Fig. 1, C). Again, the control oligonucleotides 4626 and 4258 did not substantially affect Bcl-2 and Bcl-xL protein levels and, in all further experiments, only oligonucleotide 4626 was used as a control. Bcl-xL migrated as a doublet, and both bands were diminished by antisense treatment. Bcl-xS, a pro-apoptotic splice variant of the bcl-x gene, was not detectable under these conditions (data not shown).

Inhibition of Tumor Cell Growth by Oligonucleotide 4625 in Lung, Breast, Colorectal, and Prostate Carcinoma Cells and in Malignant Melanoma Cells

Bcl-2 and Bcl-xL are survival factors protecting cells from apoptosis induced by a variety of exogenous and endogenous stimuli. Having demonstrated the bispecific activity of oligonucleotide 4625 on the level of bcl-2 and bcl-xL mRNA and protein, we further examined its ability to inhibit the *in vitro* growth of a series of cell lines derived from lung, breast, colorectal, and prostate carcinomas and from malignant melanoma. As measured in MTT assays 72 hours after the start of transfection, oligonucleotide 4625 inhibited the growth of all cell lines in a dose-dependent manner, with IC_{50} values ranging from 90 to 730 n*M* (Fig. 2). At a concentration of 800 n*M,* the levels of inhibition ranged from 57% for LoVo cells to 93% for Colo-320 cells. With the exception of LoVo cells ($P = .150$), the inhibition of cell growth was statistically significant (P values ranging from .024 to .004) as compared with control oligonucleotide 4626. The nonspecific cytostatic effect of oligonucleotide 4626 was comparatively low. The optimal antisense concentration, which is defined by a maximum difference in cell growth inhibition achieved with oligonucleotides 4625 and 4626, ranged from 400 to 600 n*M*.

Induction of Tumor Cell Death and Apoptosis by Oligonucleotide 4625 in Lung, Breast, Colorectal, and Prostate Carcinoma Cells and in Malignant Melanoma Cells

To investigate whether cell growth inhibition was due to the induction of cell death and, more specifically, to apoptosis, we analyzed propidium iodide uptake in unfixed cells and DNA content in permeabilized cells by flow cytometry 72 hours after the start of transfection with 600 n*M* oligonucleotides. Antisense treatment induced death in all nine cell lines, although at different rates (Fig. 3, A). Uptake of propidium iodide was highest in A-375 cells (77%), LoVo cells (72%), and Colo-320 cells (65%) and less pronounced in SW2 cells (36%) and PC-3 cells (32%). The effect of oligonucleotide 4625 was statistically significantly stronger when compared with that of the control oligonucleotide 4626 (P values between .010 and .001). Cell death was mainly due to the induction of apoptosis (Fig. 3, B and C). After antisense treatment, a large fraction of cells with typical sub-Go/G_1 DNA content indicative of apoptosis was observed in Colo-320 cells (64%), A-375 cells (61%), and LoVo cells (51%). Cells with fragmented DNA were less frequent in the SW2 (20%) and PC-3 (8%) cell lines. Differences between antisense and control treatment were statistically significant (P values between .001 and .009) except for PC-3 cells ($P = .053$). Further proof that cell death induced by oligonucleotide 4625 treatment mainly occurred by apoptosis was provided by Hoechst staining of nuclei, which revealed intense nuclear fragmentation and condensation (left inset in Fig. 3, C). With the exception of LoVo cells, which showed a strong response to antisense treatment in the cell death assays but were less responsive when examined in MTT assays, these data reflected the results from the growth-inhibition assays mentioned above. Treatment with the control oligonucleotide 4626 decreased the number of viable cells to a much lesser extent.

In Vivo Antitumor Activity of Oligonucleotide 4625 Against Breast and Colorectal Carcinoma Xenografts

To determine whether the potent cell death-inducing effect of antisense oligonucleotide 4625 observed *in vitro* translated into therapeutic efficacy *in vivo,* we investigated its activity in nude mice bearing established subcutaneous MDA-MB-231 breast carcinoma or Colo-320 colorectal carcinoma xenografts. Oligo-

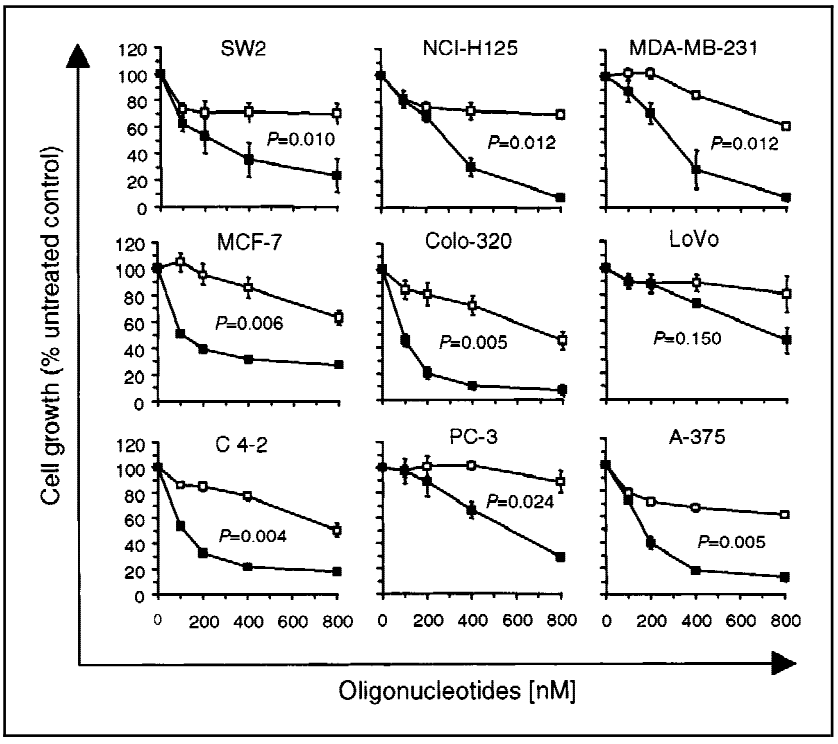

Fig. 2. Inhibition of tumor cell growth in lung carcinoma (SW2 and NCI-H125), breast carcinoma (MDA-MB-231 and MCF-7), colorectal carcinoma (Colo-320 and LoVo), prostate carcinoma (PC-3 and C4–2), and malignant melanoma (A-375) cell lines. Cells were treated for 72 hours with increasing concentrations of antisense oligonucleotide 4625 (**solid symbols**) or control oligonucleotide 4626 (**open symbols**), and cell growth was measured in quadruplicate by use of the MTT [i.e., 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reagent. Data represent the mean and 95% confidence interval (n = 3). P values were determined with the use of analysis of variance for repeated measurements.

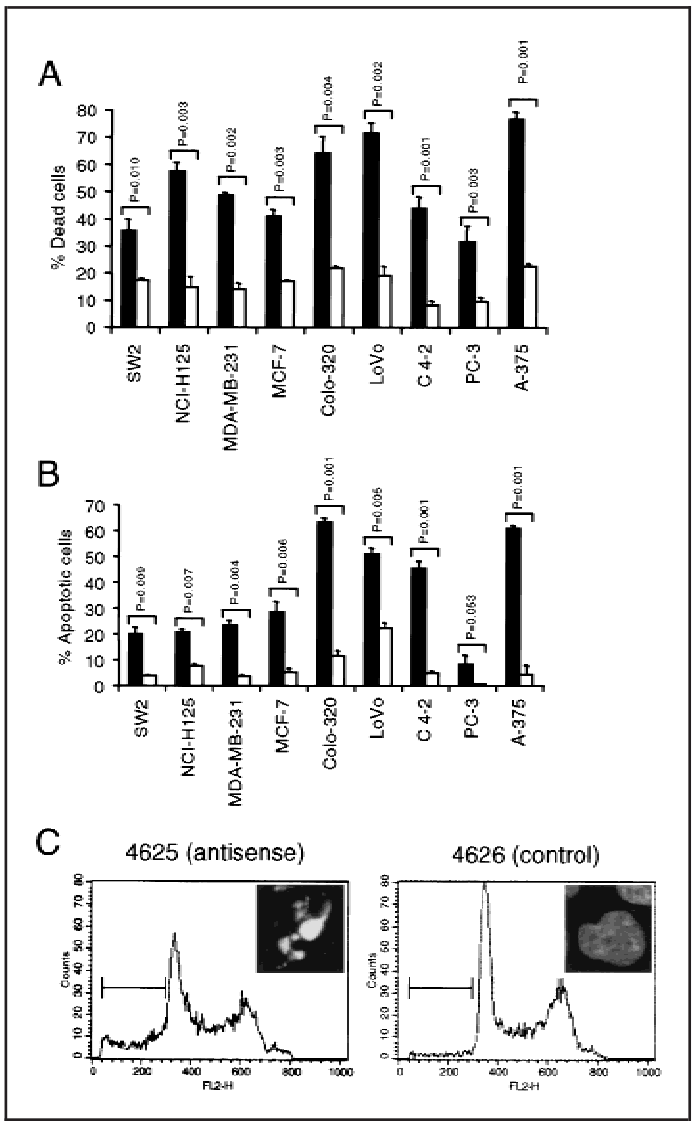

Fig. 3. Induction of tumor cell death and apoptosis in lung carcinoma (SW2 and NCI-H125), breast carcinoma (MDA-MB-231 and MCF-7), colorectal carcinoma (Colo-320 and LoVo), prostate carcinoma (PC-3 and C4–2), and malignant melanoma (A-375) cell lines. Cells were treated for 72 hours with 600 n*M* antisense oligonucleotide 4625 (**solid symbols**) or control oligonucleotide 4626 (**open symbols**). Cell death (**A**) and apoptosis (**B**) were assessed on the basis of propidium iodide uptake into unfixed or ethanol-fixed cells, respectively, by use of a flow cytometer. Data represent the mean and 95% confidence interval of three determinations. Paired *t* tests were performed to determine statistical significance. **C**) Histograms illustrating the appearance of an apoptotic sub-G_0/G_1 cell population upon treatment with antisense oligonucleotide 4625; **insets:** Hoechst stainings of nuclei confirming the apoptotic morphology of antisense-treated cells. Representative examples of MDA-MB-231 cells from the experiment described in this legend are shown.

nucleotides were injected intraperitoneally at a dose of 20 mg/kg per day over a period of 3 weeks. Antisense treatment statistically significantly inhibited the growth of both breast carcinoma and colorectal carcinoma xenografts. At the end of the 3-week treatment period, oligonucleotide 4625 had diminished the growth of MDA-MB-231 xenografts (Fig. 4, A) by 51% (95% CI = 28% to 74%) (P = .016) and of Colo-320 xenografts (Fig. 4, B) by 59% (95% CI = 44% to 74%) (P<.001) as compared with control oligonucleotide 4626. There was no statistically significant difference in the growth of untreated and control oligonucleotide 4626-treated tumors (P = .65 and P = .78, respectively). No signs of treatment-related toxicity to the animals, such as inflammation, bleeding, hepatomegaly and splenomegaly, or weight loss, were observed over the course of the study.

Inhibition of Bcl-2 and Bcl-xL Protein Expression and Induction of Apoptosis by Oligonucleotide 4625 in Tumor Xenografts

To provide evidence that the antitumor activity of oligonucleotide 4625 was due to its ability to downregulate Bcl-2 and Bcl-xL *in vivo,* we analyzed tumor xenografts treated for 2 weeks as described above by immunohistochemistry. For this purpose, MDA-MB-231 breast carcinoma xenografts were chosen because they express Bcl-2 and Bcl-xL at readily detectable levels but no splice variant Bcl-xS (data not shown). Fig. 5, A–D, shows a representative tumor xenograft in which Bcl-2- and Bcl-x-positive cells were heterogeneously distributed across the tumor sections. Whereas large areas with intense staining for Bcl-2 and Bcl-x were present in control treated tumors, antisense-treated tumors showed markedly less intense staining for both proteins in the majority of the cells, and this finding did not depend on the cell density (Fig. 5, A–D). Tumor sections processed with secondary and tertiary antibodies alone were used as negative controls (Fig. 5, E and F). Fig. 5, G, shows an immunoblot of whole-tumor lysates from representative tumor xenografts confirming the inhibition of target protein expression shown by immunostaining. The lower inhibitory effect on Bcl-xL expression might be due to the presence of mouse stromal and endothelial cells in the tumor lysate and the fact that, in contrast to Bcl-2, available antibodies do not discriminate between human and mouse Bcl-xL.

To determine whether oligonucleotide 4625 induced apoptosis in the tumors, we performed TUNEL staining 1 week after the start of treatment to detect the presence of apoptotic nuclei *in situ.* Although in all tumors few isolated TUNEL-positive nuclei were visible, oligonucleotide 4625 antisense-treated tumors clearly showed more such apoptotic nuclei (Fig. 5, H) than did control oligonucleotide 4626-treated tumors (Fig. 5, I). Thus, as expected from its proposed mechanism of action, antisense oligonucleotide 4625 reduced Bcl-2 and Bcl-xL protein levels and induced apoptosis in the tumor xenografts.

DISCUSSION

The bcl-2 or bcl-xL antisense compounds currently under clinical investigation are specific for either bcl-2 or bcl-xL, a property limiting their therapeutic efficacy in the frequent cases of advanced tumors where both cell death antagonists are expressed. The rationale behind the antisense approach described in our study was the use of an antisense oligonucleotide with the ability to simultaneously downregulate bcl-2 and bcl-xL expression. This property is likely to be of therapeutic benefit and may have a broader applicability in cancer therapy compared with the bcl-2- and bcl-xL-monospecific oligonucleotides described so far. We have recently designed the bcl-2/bcl-xL-bispecific antisense oligonucleotide 4625 and reported its ability to simultaneously inhibit the expression of bcl-2 and bcl-xL and induce apoptosis in small-cell lung carcinoma and lung adenocarcinoma cells *(25).* Here, we describe the use of this bispecific antisense compound to inhibit the growth and induce apoptosis in tumor cells of diverse histologic origins *in vitro* and *in vivo.* We have

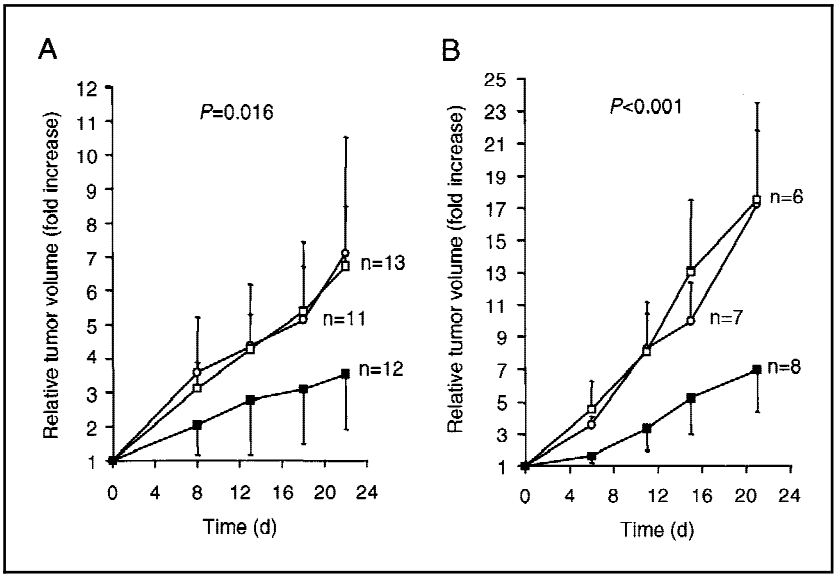

Fig. 4. Growth inhibition of tumor xenografts. Nude mice bearing established, subcutaneously growing MDA-MB-231 breast (**A**) or Colo-320 colorectal (**B**) carcinoma xenografts either remained untreated (**open circles**) or were treated with 20 mg/kg per day of antisense oligonucleotide 4625 (**solid squares**) or control oligonucleotide 4626 (**open squares**) administered intraperitoneally over a period of 3 weeks. Tumor size was measured, and the relative increase in tumor volume was calculated at the time points indicated. Data represent the mean and 95% confidence interval (n = number of animals per group). *P* values were determined with the use of analysis of variance for repeated measurements.

tested nine cell lines derived from lung, breast, colorectal, and prostate carcinomas, representing the four most prevalent and malignant tumors, as well as cells from malignant melanoma. These cell lines express distinct levels of Bcl-2 and Bcl-xL that were representative of the respective tumor types *(28–32).*

As shown by conventional reverse transcription–PCR and real-time PCR, oligonucleotide 4625 efficiently inhibited bcl-2 and bcl-xL mRNA expression in MDA-MB-231 breast adenocarcinoma cells in a dose-dependent manner. Northern blot analysis yielded identical results for the bcl-xL mRNA, whereas the inhibitory effect of oligonucleotide 4625 on bcl-2 mRNA expression could not be evaluated, since the signal was masked by strong background corresponding to the 28S rRNA (data not shown). As expected from its complementarity to the target sequences (no mismatch to bcl-2 and three mismatches to bcl-xL), the IC_{50} value for inhibiting mRNA expression was higher for bcl-xL than for bcl-2 (500 n*M* versus 250 n*M*). However, 900 n*M* oligonucleotide 4625 reduced both bcl-2 and bcl-xL mRNA levels to a similar extent, suggesting that the 2′-MOE-modified nucleotide building block could indeed largely overcome the three mismatches to bcl-xL to yield a truly bcl-2/bcl-xL-bispecific antisense compound. On the protein level, oligonucleotide 4625 was effective equally on Bcl-2 and Bcl-xL already at a concentration of 600 n*M,* a finding possibly reflecting differences in the half-lives of these proteins. In all tumor cell lines tested, treatment with 600 n*M* antisense oligonucleotide 4625 resulted in cell death, which was mainly due to the induction of apoptosis. The quantitative differences in the overall rate of cell death and apoptosis observed in some cell types suggest that cell death did not always occur by classic apoptosis. These differences may also have been due to the fact that, at more advanced stages, the apoptosis process cannot be distinguished from other forms of cell death *(27).* The colorectal carcinoma cell line Colo-320 and the malignant melanoma cell line A-375 were the most susceptible to antisense treatment, while the SW2 small-cell lung carcinoma cell line and the PC-3 prostate carcinoma cell line were the least susceptible. At present, we cannot exclude cell type-specific differences in oligonucleotide uptake and intracellular distribution *(33),* but the differential responsiveness of the cell lines to the inhibition of Bcl-2 and Bcl-xL expression suggests differences in the way in which they respond to changes in the life–death rheostat maintained by pro- and anti-apoptotic Bcl-2 family members. We further measured Bcl-2, Bcl-xL, and Bax levels by western blotting in all the cell lines used in this study (data not shown), but we could not establish a positive or negative association between the basal levels of these proteins and the results obtained in the cell viability and apoptosis assays. In view of the complexity of the apoptosis machinery and the fact that a great number of pro- and anti-apoptotic proteins are engaged in apoptosis regulation, this finding is not surprising. Intraperitoneal injection of oligonucleotide 4625 statistically significantly inhibited the growth of established subcutaneously growing MDA-MB-231 breast and Colo-320 colorectal carcinoma xenografts in nude mice. The injected dose of 20 mg/kg per day of oligonucleotide 4625 was chosen because it proved to be both effective and well tolerated in a study with a similar type of antisense oligonucleotide targeting the C-raf kinase *(34).* The antitumor effect of oligonucleotide 4625 was associated with a reduction in Bcl-2 and Bcl-xL proteins in the carcinoma xenografts, as demonstrated by immunostaining *in situ* and western blot analysis of whole-tumor lysates. The observation that the overall cell density on the tumor sections was somewhat lower in the antisense-treated than in the control-treated xenografts may be the result of increased apoptotic cell loss and the subsequent replacement of tumor tissue by connective tissue in the antisense-treated tumors. *In situ* TUNEL staining indeed revealed that tumors from mice treated with oligonucleotide 4625 showed clearly more apoptotic cells than control tumors from mice treated with oligonucleotide 4626. GenBank analysis revealed that oligonucleotide 4625 has more than six mismatches to the murine bcl-2 and bcl-xL mRNAs. Thus, despite the promising antitumor effect achieved with our antisense compound, we cannot draw conclusions regarding toxicity related to the reduction in Bcl-2 and Bcl-xL in normal tissues. In recent clinical studies *(35,36),* however, a bcl-2-monospecific antisense oligonucleotide revealed only minimal side effects, which were attributed mainly to the biochemical properties of the oligonucleotide's phosphorothioate backbone.

In a recent study *(37),* support was provided for the hypothesis that high-level expression of Bcl-2 and Bcl-xL confers a chemoresistant phenotype on tumor cells. Attempts to compare the expression of Bcl-2 or other anti-apoptotic Bcl-2 family members in tumor cells before treatment and at the time of relapse have demonstrated an apparent increase in Bcl-2 and a survival advantage for cells containing higher Bcl-2 levels *(38).* The complexity of dealing with a great number of proteins in-

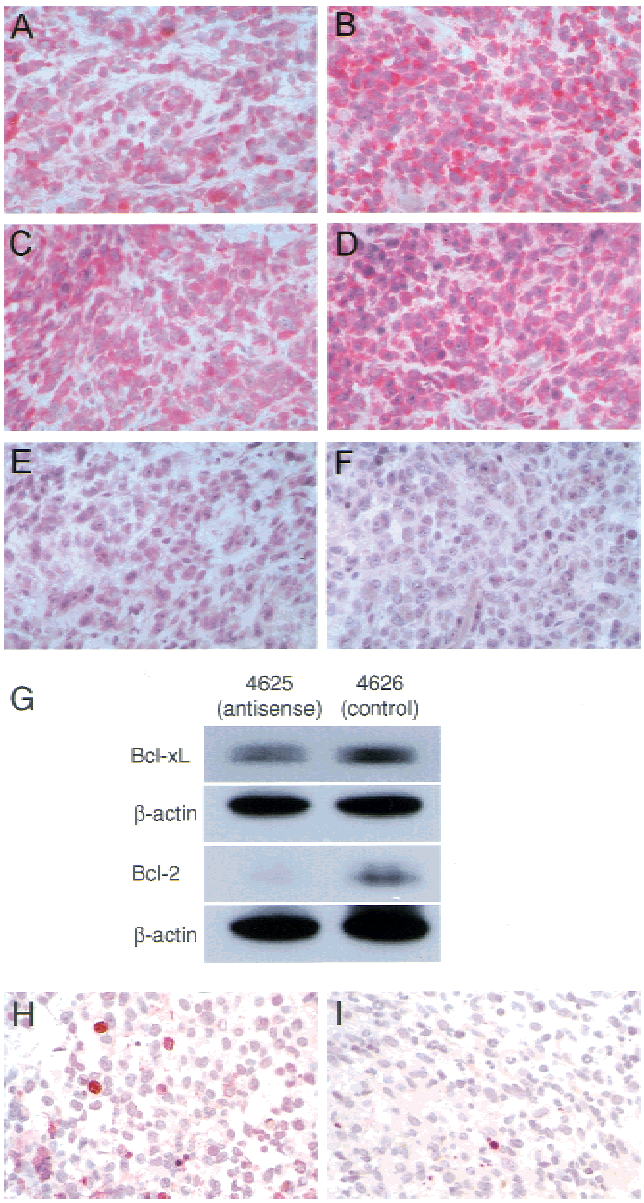

Fig. 5. Inhibition of target protein expression and induction of apoptosis in tumor xenografts. Nude mice bearing established, subcutaneously growing MDA-MB-231 breast carcinoma xenografts received antisense oligonucleotide 4625 (**A, C, E, G,** and **H**) or control oligonucleotide 4626 (**B, D, F, G,** and **I**) administered as described in the legend to Fig. 4. Tumors were dissected 2 weeks after the start of treatment, stained for Bcl-x (**A** and **B**) or Bcl-2 (**C** and **D**) by immunohistochemistry, and counterstained with hemalum. Tumor sections processed with secondary and tertiary antibodies alone were used as negative controls (**E** and **F**). Original magnification ×125. **G**) Immunoblotting of tumor lysates was performed as described in the "Materials and Methods" section to quantify the levels of Bcl-2 and Bcl-xL in antisense-treated and control treated tumors. Bands from one representative tumor per group are shown. Bcl-xL migrated at 31 kilodaltons (kd), Bcl-2 at 28 kd, and β-actin at 48 kd. TUNEL (i.e., terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-digoxigenin nick end labeling) staining was performed to detect apoptotic cells in antisense oligonucleotide 4625-treated tumors (**H**) or in control oligonucleotide 4626-treated tumors (**I**) 1 week after the start of treatment. Sections from one representative tumor per group are shown. Original magnification ×125.

volved in the apoptosis machinery that serve opposing functions limits the use of Bcl-2 and Bcl-xL as independent prognostic factors and suggests the examination of other proteins, such as the pro-apoptotic Bcl-2 family members. This issue, however, has not always been addressed consistently; probably, for this reason, clinical correlative studies have not uniformly demonstrated an adverse prognostic role for Bcl-2, and some have even shown that overexpression of Bcl-2 in tumors was associated with a favorable prognosis *(39–41).* Another important issue arises from the fact that, apart from the anti-apoptotic protein Bcl-xL, the bcl-x gene also encodes for pro-apoptotic Bcl-xS by alternative splicing of the bcl-x pre-mRNA *(42).* Although Bcl-xL seems to be the prevalent form in tumor cells and the relevance of Bcl-xS as a tumor suppressor is rather unclear *(43),* the potential to target the bcl-xS mRNA in tumor cells could be a fundamental drawback of the bcl-x antisense oligonucleotides described so far. This drawback may not apply to oligonucleotide 4625 for the following reasons: 1) The identified high homology region on the bcl-xL mRNA is localized at a splice junction site of bcl-x, and this sequence occurs neither in the mRNA of Bcl-xS nor in the mRNAs of other pro-apoptotic Bcl-2 family members; and 2) in the bcl-x pre-mRNA, the sequence targeted by oligonucleotide 4625 is interrupted by an intron, excluding the possibility that this oligonucleotide hybridizes to the bcl-x pre-mRNA.

In summary, our results demonstrate that state-of-the-art antisense technology offers a powerful approach to simultaneously target the expression of the two major anti-apoptotic proteins Bcl-2 and Bcl-xL with a single, rationally designed oligonucleotide. The functional advantage of oligonucleotide 4625's bispecificity and its broad activity against tumor cells of diverse histologic origins suggest the use of this antisense compound in cancer therapy.

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NOTES

O. Gautschi and S. Tschopp contributed equally to this work.

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